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Technical Papers
OF THE U.S. FISH AND WILDLIFE SERVICE

COMPLETED

ORIGINAL

85. Immune Response and Antibody Characterization of the Channel Catfish (*Ictalurus punctatus*) to a Naturally Pathogenic Bacterium and Virus

UNITED STATES DEPARTMENT OF THE INTERIOR
FISH AND WILDLIFE SERVICE

(40)

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Technical Papers
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85. Immune Response and Antibody Characterization of the Channel Catfish (*Ictalurus punctatus*) to a Naturally Pathogenic Bacterium and Virus

By Charles M. Heartwell III

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IMMUNE RESPONSE AND ANTIBODY CHARACTERIZATION OF THE CHANNEL CATFISH *(ICTALURUS PUNCTATUS)* TO A NATURALLY PATHOGENIC BACTERIUM AND VIRUS

by

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ABSTRACT

Inoculation of channel catfish (*Ictalurus punctatus*) with *Chondrococcus columnaris* cells and channel catfish virus resulted in a rapid primary response to both antigens. The secondary response to the bacterium was rapid but weaker than that often seen in warmblooded animals, whereas the secondary response to virus was weak, variable, and of relatively short duration.

Analysis of total serum proteins and selected isozyme systems by acrylamide gel electrophoresis revealed differences between normal and experimental fish serums in the number of serum protein components and in the number of bands of acid phosphatase, esterase, and lactic acid dehydrogenase isozymes.

Specific antibody activity of the immune serums occurred in the macroglobulin serum fraction. The macroglobulin fraction was further separated into two antibody populations by anion exchange chromatography. Antibody activity was diminished by 30 minutes exposure to 50 C, and reduction with 2-mercaptoethanol and alkylation markedly lowered antibody activity. The partly purified antibodies had a molecular weight of 800,000 to 900,000 daltons. Immunoelectrophoresis of antibacterial serums produced two specific immunoglobulin arcs of beta to gamma mobility, and antiviral serums produced one immunoglobulin arc of alpha mobility.

Partial antibody characterization indicates that specific channel catfish immunoglobulins are macroglobulins with characteristics like those of most fish immunoglobulins and similar to those of human immunoglobulin-M.

INTRODUCTION

Classical immunology was developed for man and later for warmblooded domestic animals. The immune mechanisms of poikilothermic vertebrates received only cursory attention until the 1960's, when an interest in comparative immunology began to develop as an integral part of knowledge of the phylogeny of the immune response.

Studies of fishes have shown that versatility of the response increased with evolutionary development. Basic immunocompetence of fishes has been established for only a few representatives of major orders. Heuzeroth et al. (1973) stated that immunoglobulin differences between the orders of fishes are as great as those between the classes of vertebrates.

Application of immunological principles to cultured fishes has the potential for providing

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the same advantages to fish husbandry that it has provided to warmblooded animal husbandry and to man. The establishment of basic immunological competence of specific fish species and the application of immunization methods to fishes are still in the investigative stages. Basic serological principles are being employed to a limited extent, but protective immunology is not a routine part of fish husbandry.

Most immunological research has been conducted on the salmonids and cyprinids—the salmonids because they are economically important as food and sport fishes in the United States, and the cyprinids because they are inexpensive, easily obtained, and easily maintained. Recent intensification of propogation of channel catfish, *Ictalurus punctatus*, has brought with it disease problems and the need for expanded research.

The limited immunological and serological research that has been performed on channel catfish has emphasized responsiveness of immunoglobulin systems to certain viral antigens (McGlamery et al. 1971; Plumb 1973b). There has been no research on the kinetics of the immune response to antigens of natural pathogens, on antibody characterization, or on enzymograms as possible diagnostic aids.

The present study followed the response of channel catfish to representative natural pathogens—a bacterium, *Chondrococcus columnaris*, and a virus, channel catfish virus (CCV); (Fijan et al. 1970). The study was designed to develop a humoral antibody response to the two antigens, to follow the kinetics of the immune response, and to partially characterize the antibodies produced.

LITERATURE REVIEW

Immune Response

Reviews of the immune response in fishes by Post (1963), Ridgeway et al. (1966), Mohler (1969), Cushing (1970), and Snieszko (1970) revealed a paucity of research until recent years. This is especially true of the immune response of warmwater fishes to natural pathogens.

Fish immunological literature is varied as to species of fish, type of antigen, environmental conditions, and research goals. There appears to be sufficient homogeneity in research findings, however, to permit certain generalities:

1. The immune response of bony fishes is directly dependent on environmental temperature and the nature of the antigen.
2. The species of fish and route of antigen injection are of secondary importance in the mechanism of the immune response.
3. Teleost fishes are capable of a rapid and strong primary response under optimum conditions for the immune response.
4. The secondary immune response in fishes can be highly variable and is often weaker than that found in mammals.
5. The primary and secondary responses of fishes are often shorter than those of mammals.
6. Oral immunization of fishes has met with only limited success.

Since major advances in fish immunology began in approximately 1966 (Cushing 1970), the following chronological literature review emphasizes the period 1966-73.

Post (1963) studied the immune response of 19-mo-old rainbow trout (*Salmo gairdneri*) to the bacterium *Aeromonas hydrophila* at 15.5 C with several immunization regimes. Monthly injections of bacteria in saline for 7 mo yielded maximum agglutination titers of 1:32; antigen injections in Freund's Complete Adjuvant (FCA) yielded maximum titers of 1:64; and oral immunization, daily for 9 mo, yielded maximum agglutination titers of 1:8. Titers of 1:4 yielded protection to LD_{90} challenge doses of bacteria. Individuals receiving bacteria suspended in saline showed a definite titer loss 5 mo after the last antigen inoculation, but about one-half remained in the immune class for 7 mo after the last inoculation. About 50% of the fish given bacteria suspended in FCA were also in the immune class 7 mo after inoculation.

Ambrosius and Schäker (1964), who immunized carp (*Cyprinus carpio*) with swine serum, showed that rate of antibody response increased with temperature; titers were highest in 20 to 40 days at 20 C, the highest temperature tested. A secondary injection at 14 wk produced an anamnestic response.

Clem and Sigel (1963), who used multiple injections of PR-8 virus, produced 50-day titers of 1:40 to 1:640 in margates (*Haemulon album*). Injection with *Salmonella paratyphi* B gave titers of 1:80 in 58 days, and bovine serum albumin (BSA) produced passive hemagglutination (PHA) titers of 1:50,000 in 50 days.

Papermaster et al. (1964) immunized several species of fishes with bacteria, bacteriophage, hemocyanin, and BSA. Black bullheads (*Ictalurus melas*) immunized with bacteriophage T_2 at 10 C and 20 C exhibited circulating antibody in 14 days at 10 C and in 4 days at 20 C. Largemouth bass (*Micropterus salmoides*) formed precipitating and hemagglutinating antibody to hemocyanin injections. These authors found progressive levels of immunologic reactivity with increasingly differentiated and complex immunologic mechanisms present as the phylogenetic scale was ascended from hagfish (*Myxine glutinosa*) to the teleosts.

In rainbow trout immunized by intraperitoneal (i.p.) injection of *Aeromonas salmonicida*, Spence et al. (1965) found maximum titers of 1:640 in 2-yr-old trout 10 wk after multiple primary injections and in 3- and 4-yr-old trout after 17 wk. Oral immunization of juvenile coho salmon (*Oncorhynchus kisutch*) with *A. salmonicida* for 14 wk did not yield significant resistance to furunculosis.

Golden shiners (*Notemigonus crysoleucas*) held at 20–26 C produced increasing amounts of antibody to i.p. injections of *Aeromonas liquefaciens* over a 59-day test period and reached a peak mean titer of 1:1,280 (Summerfelt 1966). Clem and Sigel (1966), who immunized margates and gray snappers (*Lutjanus griseus*) with BSA, observed PHA titers of 1:10,000 to 1:40,000 in 30 to 40 days; secondary injections resulted in titers of 1:160,000 to 1:650,000.

Watson et al. (1968) found that golden shiners and goldfish (*Carassius auratus*) inoculated with two bacterial fish pathogens (*A. liquefaciens* and *Streptococcus OX39)* reached maximum agglutination titers of 1:1,024 after 12 to 13 days.

Carp of 250–300 g body weight, held at 25 C and given a single intramuscular (i.m.) injection of BSA in FCA, responded with PHA titers in excess of 1:20,000 in 28 days (Avtalion 1969). A second group of carp given the same injection and transferred to 12 C after detectable antibody synthesis had begun, corresponded closely in time of response and peak titers to fish held constantly at 25 C. Avtalion et al. (1970) later corroborated the finding that the immune response was temperature dependent among carp. Fish held at 25 C had an immune response similar to that of fish in the earlier work, but carp held at 12 C and 14 C produced no measurable antibody response in 63 days.

Teleosts (margate, snapper, grouper) injected with 2,4-dinitrophenylated bovine gamma globulin and 2,4-dinitrophenylated bovine serum albumin showed higher agglutination titers than did elasmobranches (sharks) (Clem and Leslie 1969).

Yu et al. (1969) demonstrated the formation of antibody that neutralized Infectious Pancreatic Necrosis Virus (IPNV) 3 wk after injection of viable IPNV in blue gourami (*Trichogaster trichopterus*) maintained at 26–28 C.

Adult goldfish were held at 20, 25, and 30 C and immunized with multiple injections of BSA by Trump and Hildemann (1970). After primary antibody titers peaked, as determined by PHA, the fish were given an additional injection to follow the secondary response. Antibody was detected at 20 C in 17 days and reached a peak titer of 1:32 by 38 days postinoculation. Antibody was detected in 7 days at 25 and 30 C and peaked in 20 days at 25 C and 13 days at 30 C. The peak titers were 1:128 at 25 C and 1:8 at 30 C. The rapidity of the secondary response was related to temperature. The secondary response began more quickly and was much stronger at 25 C than at 20 or 30 C.

Frenzel and Ambrosius (1971), who immunized carp with dinitrophenol (DNP)-BSA at 25 C, detected antibodies in 10 days which reached a maximum titer 50 days after injection. Secondary injections resulted in an anamnestic response, but neither primary nor secondary titers were large.

Marchalonis (1971) immunized goldfish with i.p. and i.m. injections of human immunoglobulins in FCA, and carp with a single i.p. injection of *Limulus* sp. haemocyanin in FCA. The presence of antibodies was determined by PHA and double diffusion in agar gel. Goldfish showed specific PHA titers up to 13 $\log_2$ units 28 days postimmunization. A second inoculation on day 29 yielded no secondary response.

Shelton and Smith (1970) immunized carp with multiple i.m. injections of DNP-BSA and sheep red blood cells. Maximum PHA titers against DNP-BSA of 1:8,000 were observed 14 days after final injection. Maximum titers against the sheep red blood cells were 1:10,000, 16 days after the final injection.

Evelyn (1971) immunized 1-to 3-yr-old sockeye salmon (*Oncorhynchus nerka*) with a single i.p. injection of the kidney disease bacterium in FCA at 12–15 C. The peak primary response occurred in 90 days, and a single secondary injection produced an anamnestic response, with a maximum titer of 1:10,240, 90 days later.

One- and two-yr-old carp held at 18 to 20 C by Goncharov (1971) were immunized with multiple i.p. injections of suspended *Aeromonas punctata*. He achieved the highest titers using 10 primary injections at 10-day intervals and a secondary injection 75 days after the last primary injection.

Active immunity to *Chondrococcus columnaris* disease was established in coho salmon at 18 to 20 C with oral vaccine and i.p. and subcutaneous injections of antigen (Fujihara and Nakatani 1971). Orally immunized fish experienced 40% less mortality than nonimmunized control fish when challenged with viable *C. columnaris*. Salmon receiving i.p. injections of antigen in FCA developed the highest titer of 1:5,120 in 12 wk.

Cisar (1972) immunized juvenile coho salmon with a single i.p. injection of killed *A. salmonicida* whole cells and obtained maximum agglutination titers of 1:20,480, 10 wk after injection. Natural agglutination titers of 1:20 were present in most fish before immunization and were as high as 1:60 in the control fish at the end of the study. These titers were probably due to either increased natural agglutinins or exposure to the bacteria during the experiment.

One group of rainbow trout injected by Dorson (1972) with a protein-coupled hapten in FCA had hemagglutination (HA) titers of 1:10,000, 2 mo postimmunization; 20 to 40% of a second group injected with viable bacteriophage exhibited a response. Everhart (1972) found that goldfish immunized with BSA and haptens coupled to BSA had peak PHA titers of 1:320 to 1:10,000 in 70 days at 24 to 28 C.

Kurashige et al. (1972) immunized rainbow trout held at 17 to 18 C with flagellae from *Salmonella tennessee*. The immune response was detectable in 2 wk by slide agglutination, peaked at 6 to 7 wk and did not decline until 20 wk postimmunization.

Thomas et al. (1972) demonstrated anti-phage properties in five of nine adult thornbacks (*Platyrhinoides triseriata*) after a single intercardiac injection of bacteriophage. Maximum response occurred in 4 days at 19 C.

Vladimirov (1972) studied the immune response of Ropsha carp to natural and experimental infections of the viral agent of carp red rot; he found that fish held at 20 to 22 C showed a measurable immune response in 8 days. Recovery from the disease resulted in a high degree of immunity for 1 yr after infection. Maximum serum antibody levels, on the basis of several assay systems, were 1:4,096 for HA, 1:2,048 for PHA, and 1:512 for bacterial-viral agglutination.

Harris (1973) compared the immune response of dace (*Leuciscus leuciscus*) at 2, 5, 10, and 18 C. Time to antibody production increased as temperature decreased.

Hines and Spira (1973), who exposed mirror carp to controlled numbers of viable *Ichthioptherius multifiliis* at 18 to 20 C, found no reinfection for 8 mo in fish that recovered from an active infection. Serum antibody titers ranged from 1:64 to 1:1,024, 7 days after recovery from infection (based on the parasite immobility test).

Jørgensen (1973) immunized rainbow trout held at 17 to 21 C with IPNV. The immunization titers peaked at 12 wk with a maximum plaque reduction titer of 1:24,000. Nonimmunized control fish and preimmunization fish had plaque reduction titers ranging from 1:400 to 1:2,800.

Immunological Studies on Catfishes

Research reports of particular interest to the present study are those in which catfishes have been used. Chuba et al. (1968) and Wiener et al. (1968) studied the response of the brown bullhead (*Ictalurus nebulosus*) to human secretor materials. Both of these experiments showed that the test animals responded with high specific HA titers to the major human blood group substances.

Australian freshwater catfish (*Tandanus tandanus*) immunized with human secretor materials responded with high titered specific hemagglutinating and precipitating antiserums (Baldo and Boettcher 1971). Most of the experimental fish had natural agglutinins to one or more of the human secretor materials. Titers could sometimes be increased by injecting the secretor material. The species possessed a remarkable ability to distinguish between the human A, B, and H blood glycoproteins.

Australian catfish (*Tachysurus australis*) inoculated with BSA and formalinized *Salmonella enteritidis* antigen reached peak indirect hemagglutination titers of 1:2,048, 56 days after injection (DiConza and Halliday 1971). Titers showed a noticeable decrease about 19 wk after final injection. Fish injected with the bacterium had titers up to 1:512 on day 42 but were not followed beyond this time.

McGlamery et al. (1971) studied the immune response of channel catfish to BSA and vesicular stomatitis virus from 24 to 30 C. A single injection of BSA in FCA was sufficient to produce an antibody response of 1:2,560 in 4 wk, whereas the 50% endpoint of vesicular stomatitis virus in FCA at 4 wk postinjection was 1:640.

Channel catfish fingerlings held at 28 C developed mean titers of 1:4,337 by slide agglutination 3 wk after injection with *Chondrococcus columnaris* whole cell antigen (Anonymous 1971).

Plumb (1973a, 1973b) investigated some biological aspects of channel catfish virus disease. Catfish at 22 to 24 C were injected i.p. with viable virus. Serum was assayed at 30-day intervals for virus neutralization activity. A positive immune response was detectable 30 days postinjection, and the maximum primary titers, a neutralization index of 3,162 were evident at 60 days postinjection. The anamnestic response was rapid, reached maximum titer in 30 days, and was greater than the initial response.

Gel Electrophoresis

The advent of electrophoresis in starch gels by Smithies (1959) and in polyacrylamide gels by Ornstein (1964) and Davis (1964) provided clinical chemistry, genetics, and related disciplines with an exceptional research and diagnostic technique for protein separation and analysis.

Among those who adapted the disc electrophoretic technique to enzyme analysis were Markert and Moller (1959), Hunter and Burstone (1960), Allen (1961), Hodson et al. (1962), and Allen et al. (1965). A 1961 symposium on multiple molecular forms of enzymes, published by the New York Academy of Sciences, consolidated earlier information and solidified the reasoning for the clinical use of multiple enzyme separation.

The usefulness of zymograms in clinical chemistry is now well documented (Anonymous 1964; Bodansky 1958; Hauss and Leppelmann 1958; Latner and Skillen 1968; Maurer 1971; and Wolfson et al. 1958).

After development of clinical gel enzymography, geneticists began using zymograms as generic and species-specific markers for a wide range of plants and animals (Kristjansson 1963; Colwell et al. 1973; Kilgour and Godfrey 1973; and Bagster and Parr 1973).

Disc gel electrophoresis in fishery research has found its broadest application in genetics. Among the investigators who have detected isozyme differences attributed to genetics are Fujino and Kang (1968), Markert and Faulhaber (1965), Williscroft and Tsuyuki (1970), Hochachka (1966), Goldberg (1965a), Clayton and Tretiak (1971), Utter and Hodgins (1972), Morgan et al. (1973), and Eckroat (1971).

Almost no research has been done on the use of serum proteins and isozyme disc electrophoretic patterns as indicators of disease in fishes until recent years. Mulcahy (1967) detected changes from the normal in the serum protein fractions of Atlantic salmon (*Salmo salar*) with ulcerative dermal necrosis (UDN). A total of five major serum protein fractions and three minor fractions were resolved in healthy fish, whereas one major fraction was lost, two major fractions increased in intensity, and two additional minor fractions were evident in diseased salmon. Mulcahy (1969) later compared serum protein fractions of healthy and UDN infected salmon and trout from seven rivers. She concluded that protein changes in the early stages of UDN were specific and of diagnostic value for the disease. In late UDN infections, however, serum protein patterns were not as characteristic or distinct.

Mulcahy (1971) also examined the serum protein fractions of brown trout (*Salmo trutta*) injected with UDN and fractions from normal fish. Five major and three minor serum components were observed. The qualitative pattern of the fractions did not change in diseased trout but the level of protein in some of the fractions changed. These changes were not deemed of diagnostic significance, however, since analytical changes in acrylamide gels are difficult to evaluate.

Thurston (1967) compared serum protein patterns of hatchery rainbow trout subjected to several stresses and the electrophoretic pattern of hatchery versus wild rainbow trout. The total number of protein fractions ranged from 10 to 18. Protein pattern changes were attributable to stage of maturity, method and frequency of sampling, and method of capture; differences between hatchery and wild rainbow trout were significant but variability between individual fish made characterization of a "normal" disc gel electrophoretic pattern impossible. Extreme caution was recommended in interpreting protein patterns after electrophoretic separation.

Bell (1968) investigated changes in the serum glutamic oxalacetic transaminase (SGOT) level of salmon (*Oncorhynchus spp.*) fed hepatic poisions and infected with kidney disease. The SGOT levels in fish treated with hepatic poisions, as well as in salmon infected with kidney disease, were significantly higher than the levels in untreated fish.

Amend (1973) studied the pathophysiology of Infectious Hematopoietic Necrosis Virus (IHNV) disease in rainbow trout. He used acrylamide gel disc electrophoresis and starch gel electrophophoresis to examine serum LDH, esterase, peptidase, and SGOT. There was no change in esterase, SGOT, or peptidase isozymes. An LDH isozyme, designated $B^{2'}4$, showed significant increase in IHNV infected fish by the fourth day postinjection of the virus. This change was not detectable in the LDH isozyme patterns of muscle, anterior and posterior kidney, liver, spleen, or gill. The plasma LDH isozymes from rainbow trout infected with IPNV, *Vibrio anguillarium*, *Aeromonas salmonicida*, and redmouth bacteria did not show the LDH alteration evident in IHNV infected fish.

Cisar (1972) examined normal serums and anti-*A. salmonicida* serums from juvenile coho salmon on polyacrylamide gels. Normal and immune serums were resolved into 24 components. There were no obvious differences between them or between absorbed and unadsorbed normal and immune serums. Close observation of the immunoglobulin disc on polyacrylamide gels in normal and immune serums also revealed no obvious differences.

Antibody Characterization

Although the immunoglobulins of several teleost fishes have been studied, information is lacking for most members of the group.

A summary of the literature on the characterization of teleost immunoglobulins permits the following general statements.

1. The characteristics of teleost immunoglobulins depend partly on the species of fish and type of antigen used.
2. Teleost immunoglobulins are heterogeneous; most fish species produce at least two types of specific immunoglobulins.
3. Most fish immunoglobulins are macroglobulins with the characteristics of Immunoglobulin-M (IgM) of higher animals.
4. Almost no teleost demonstrates the antibody shift from heavy to light molecular weight immunoglobulins that is characteristic in mammals.
5. Fish immunoglobulins are more heat labile than most mammalian immunoglobulins, and most are susceptible to partial reduction and alkylation with 2-mercaptoethanol (ME).
6. Fish immunoglobulins exhibit a wide range of electrophoretic mobility.

Post (1963) separated rainbow trout serum proteins by their salt solubility and electrophoretic properties on paper, cellulose acetate, and starch gel. Normal serum separated into three major components, but these components did not coincide with the alpha, beta, and gamma fractions of higher animals. The antibody fraction most nearly resembled the beta-globulins and slow moving alpha-globulins of higher animals. A component equivalent to the gamma-globulin of higher animals was not observed in either normal or immune trout serum.

The studies of Ridgeway et al. (1966) indicated that the immunoglobulins of salmonid fishes are heterogeneous, both antigenically and electrophoretically. The immunoglobulin found in most serums had the mobility of gamma globulin.

Hodgins et al. (1967) separated a rainbow trout specific immune serum into three protein antigens by gel filtration and analytical ultracentrifugation. The fast immunoglobulin peak had a sedimentation coefficient of 13.2S to 13.9S, the slower peak was 10.5S to 10.7S. The immunoglobulins had the mobility of human beta-globulins. Treatment at 59 C for 30 min destroyed specific antibody activity, and treatment at 58 C for 15 to 30 min destroyed natural hemagglutinins.

Albino rainbow trout serum was separated into 12 components with immunoelectrophoresis by Anderson and Klontz (1970). Specific antibody activity occurred in the fraction which corresponded to the beta-globulin fraction of rabbit serum. No component comparable to rabbit gamma-globulin was detected. The immunoglobulin precipitin line varied in intensity and vertical position with the time of immunization. The precipitin line was most dense and nearer the center trough at peak titers.

Antiserums produced against the bacterial kidney disease organism in sockeye salmon contained antibodies which ranged in position from the immobile gamma-globulin peak to a position just behind the alpha-globulin peak (Evelyn 1971). The antibody activity was completely inactivated by exposure to 56 C for 15 min, or by treatment with mercaptoethanol.

Cisar (1972) conducted an exhaustive study on the properties of anti-*Aeromonas salmonicida* antibodies from juvenile coho salmon. Temperatures of 50 C for 30 min destroyed 50% of the antibody activity of immune serum, 55 C destroyed 75%, and 60 C destroyed more than 95%. Partial alkylation and reduction lowered the agglutination activity of immune serum by 97%, and exposure of partially alkylated and reduced immune serums to 2 Molar (M) urea resulted in a complete loss of antibody activity. Sephadex G-200 gel filtration revealed that the antibody was a macroglobulin (>200,000 mol wt).

Purified coho salmon immunoglobulin had a sedimentation value of 16.9 to 17.3S and characteristics of IgM (Cisar 1972). There was no evidence of a low molecular weight antibody in immune serums during the 20 wk of immunization. Approximate molecular weights were 73,000 to 76,000 for heavy chains and 26,000 for light chains. Electronmicroscopy of purified immunoglobulin revealed a four-armed macroglobulin shaped like a "+".

Immunodiffusion of the purified antibody from coho salmon gave a single precipitin arc (Cisar 1972). Immunoelectrophoresis did not reveal immune serum components of gamma-like mobility. Normal coho salmon serum and immune coho serum versus rabbit anti-whole coho serum showed no significant differences in precipitin arcs.

Dorson (1972) found the immune globulins of rainbow trout to be macroglobulins of beta-globulin electrophoretic mobility. Complete reduction and immunoglobulin chain separation on Sephadex G-100 yielded chains with the same mobility as human L chains and H chains. The trout macroglobulins also had features of IgM rather than Immunoglobulin-G (IgG) molecules.

Jørgensen (1973) partly characterized rainbow trout anti-IPNV serum using Sepharose 4B column chromatography, immunoelectrophoresis, and partial reduction and alkylation. The trout immunoglobulin was a macroglobulin of beta mobility. Antibody activity was destroyed by partial reduction and alkylation.

Kurashige et al. (1972) immunized albino rainbow trout with i.p. injections of *Salmonella tennessee* flagellae and found fish which were injected in the spring produced only 19S immunoglobulins during that spring, but by fall, immune serums contained both 19S and 7S immunoglobulin fractions. Spring immunoglobulins had alpha mobility but fall immune serums had alpha, beta, and gamma mobility. The 19S antibodies were inactivated at 56 C for 30 min, but the 7S antibodies were stable. The 19S immunoglobulin was resistant to 2-ME, whereas the 7S immunoglobulin was totally inactivated by 2-ME treatment.

More emphasis has been placed on the study of serum components in carp and goldfish than in any other teleosts because these two species are readily available and easy to maintain. Sorvachev et al. (1962) stated that the principal serum components of carp were albumins, beta-globulins, and gamma-globulins. Carp hyperimmunized with attenuated rubella virus showed an increase of gamma-globulin and a sharp decrease in albumin. The alpha and beta globulins also showed some decrease with immunity.

Uhr et al. (1962) injected goldfish held at 30 to 32 C with bacteriophage Φ × 174 for 5 mo. Electrophoresis showed the antibody activity was chiefly in the fraction corresponding to human gamma-globulin. The antibody activity was evenly distributed between heavy and light antibody fractions at 2 mo, and almost entirely in the light antibody fraction at 5 mo. This is the only research which has shown the shift from heavy to light immunoglobulins in fish. Early and late serums were sensitive to 2-ME treatment.

Gulyaev et al. (1966), using paper electrophoresis, compared normal serum components of several fish species with the same serum components of fish with bacterial red rot disease. The serums of wild carp, cultured carp, tench, marinka, silver carp, white amur, and perch were electrophoretically separated into albumin and beta-, alpha-, and gamma-globulins. Serum components of the different species did not migrate at the same rates. Serums of cultured carp, wild carp, and white amur with red rot disease showed an increase in the beta- and gamma-globulins and a decrease in albumins. Specific antibodies were associated with gamma-globulin fractions.

Ambrosius et al. (1967) found two distinct populations of antibodies in immunized carp. The preimmunization carp serum samples contained a protein with gamma-globulin mobility, and immune serums contained immunoglobulins of beta and alpha mobility. The immunized carp serums contained 2-ME sensitive and 2-ME resistant antibody fractions.

Watson et al. (1968) studied the immune serums of goldfish and the golden shiner to *Aeromonas liquefaciens* and *Streptococcus* OX39. The peak primary antibody titers to both pathogens occurred 12 to 15 days after inoculation. The immunoglobulins had gamma-globulin mobility.

Summerfelt (1966) also studied normal serums and anti-*A. liquefaciens* antibody of the golden shiner. Normal serum was separated into six components with paper electrophoresis. The gamma-globulin and beta-globulin fractions increased after immunization. The antibody activity was largely associated with the gamma-globulin fraction.

After immunization of carp with DNP-BSA a single immunoglobulin component was observed that resembled rabbit anti-DNP and high—affinity immunoglobulin-A (IgA) mouse myeloma proteins in cross reactivity (Smith and Potter 1969).

Shelton and Smith (1970), who purified carp antibody and studied its ultrastructure, found the immunoglobulin to be a 14.5S tetrameric molecule measuring approximately 385 Å across the span of the arms and 98 Å across the diameter of the central region.

Trump (1970) and Trump and Hildemann (1970) studied the antibody response of goldfish to BSA. Two antibody populations separated by diethylaminoethyl (DEAE) column chromatography and Sephadex G-200 filtration migrated differently with starch block electrophoresis. Antibody population number two was antigenically deficient with respect to number one, showing only partial identity on double gel diffusion. There was no dramatic shift from one population to the other during the primary or secondary response, and population one appeared before population two during the primary response.

Everhart (1972) also separated two antibody populations from immune goldfish serum which had different immunoelectrophoretic mobilities. Both purified antibodies had sedimentation coefficients of 13.2S. Goldfish antibody was found to compare favorably in titer and specificity with rabbit antibody made against the same antigen.

Heuzeroth et al. (1973) studied the antigenic similarities between carp immunoglobulins and the immunoglobulins of 22 species of fish and representatives of other vertebrate classes. He reported that the differences between the orders of fishes were as great as the differences between fishes and other classes of animals.

Marchalonis (1971) partly characterized and compared the immunoglobulins of carp and goldfish. The purified immunoglobulins of carp and goldfish had the electrophoretic mobility of gamma-globulin or beta-globulin. The antibody of the carp had slightly more cathodal mobility than that of the goldfish. Antibody activity was associated with macroglobulins and had a sedimentation coefficient of about 19S. Double-gel diffusion indicated that the carp immunoglobulin was antigenically deficient with respect to the goldfish immunoglobulin. Alkylation and reduction revealed light chains which resembled those of other vertebrates. The heavy chains had molecular weights of about 70,000, a value comparable with the mu-chains of IgM antibody in higher vertebrates.

Perch (*Perca fluviatilis*) immunized with human IgG had immunoglobulins with beta and alpha mobility (Ambrosius et al. 1967). The sedimentation coefficient of both primary and secondary antibodies was 14S to 15S and both were resistant to 2-ME treatment.

Yu et al. (1969) electrophoretically separated normal and anti-IPNV blue gourami serum into four major components comparable with the gamma-, beta-, and alpha-globulin and albumin components of rat serum. The gamma-globulin fraction contained the anti-IPNV antibodies. Thomas et al. (1972) studied immune serum of the thornback. The antibody activity was located in the macroglobulin fraction and migrated immunoelectrophoretically as the IgM found in higher vertebrates.

Among the most critical works on immunoglobulin characterization in fishes is that on marine teleosts from Florida. Clem and Sigel (1963) performed studies on margate anti-BSA serum which separated immunoelectrophoretically into three distinguishable cathodic moving proteins of gamma and beta mobility. The antiserum was stable at 56 C for 30 min. Additional studies (Clem and Sigel 1966) revealed that margates produced no 2-ME resistant antibodies during a 10-mo study. Studies on the gray snapper did reveal a 2-ME resistant anti-BSA antibody which was evident at 30 days postinjection. This resistant fraction was, however, a macroglobulin, unlike resistant molecules in higher vertebrates. The electrophoretic migration of the gray snapper antibody placed it in the same region as the antibody of the margate.

Clem and Leslie (1969) separated margate immunoglobulins into 19S and 7S fractions; both fractions had characteristics of the IgM class. Russell et al. (1970) also examined anti-DNP antibodies of the gray snapper and confirmed the presence of a macroglobulin component with a 19S sedimentation coefficient. The affinity of the snapper antibody was low and did not increase with time—a characteristic not typical of mammalian IgG.

Clem and Leslie (1969) found that purified immunoglobulins from the giant grouper (*Epinephelus itaira*) contained fractions with 6.4S (mol wt 120,000) and 15S (mol wt 900,000) sedimentation coefficients. The 15S immunoglobulin had properties similar to those of IgM, and the 6.4S fraction appeared to be either a degradation product of the 15S fraction or a genetic deletion, since it was antigenically deficient. Continued work with the giant grouper immunoglobulins showed that both the 15S and 6.4S immunoglobulins possessed gamma mobility when immunoelectrophoresed (Clem and Small 1970).

Clem (1971) continued work with the giant grouper and determined molecular weight of 120,000 and 700,000 for the fractions; both immunoglobulins had beta mobility. Alkylation and reduction followed by Sephadex G-200 gel filtration was used to characterize the polypeptide chains. The 16S immunoglobulin had H (mol wt~70,000) and L (mol wt~22,000) polypeptide chains. By comparison, the 6.4S immunoglobulin had lighter H chains (mol wt~40,000) and L chains of the same size (mol wt~22,000) as those of the 16S immunoglobulin.

The globulin fractions of several species of ictalurids have received attention. Ambrosius et al. (1967) immunized brown bullheads (*Ictalurus nebulosus*) with human IgG and whole pig serum. The immunoglobulins of the primary and secondary response were completely destroyed by treatment with 2-ME. Studies of the blood groups of North American freshwater catfishes by Kuhns and Chuba (1968) revealed that the electrophoretic mobility of the natural agglutinins was similar to that of human IgM. Sedimentation studies indicated the presence of light and heavy components, both of which were sensitive to 2-ME reduction.

The natural hemagglutinins, and whole serum and mucus immunoglobulins of the Australian freshwater catfish showed the natural hemagglutinins and specific immunoglobulins to BSA to be macroglobulins that were completely inactivated by 2-ME treatment (DiConza 1970; DiConza and Halliday 1971). The natural agglutinins were stable to a temperature of 56 C for 30 min but were destroyed at 60 C. The immunoglobulin component was separated into two fractions with DEAE column chromatography.

Acton et al. (1971), who purified anti-*Salmonella typhosa* "O" antibodies from channel catfish serum, found they were tetrameric globulins with a molecular weight of 600,000—considerably lower than that of mammalian IgM.

MATERIALS AND METHODS

Experimental Fish

Three-year-old channel catfish (*Ictalurus punctatus*) from the Harrison Lake National Fish Hatchery, Charles City, Va., and the Leetown National Fish Hatchery, Leetown, W. Va., were used as test animals.

All fish were coldbranded (Everest and Edmundson 1967) and held in individual pens (33 × 33 × 51 cm) in an isolated warmroom holding facility at the Eastern Fish Disease Laboratory. The channel catfish were separated into three groups (I, II, and III) for experimentation. Within each group were included experimental controls, fish which received bacterial antigen, and fish which received viral antigen. The trials were conducted separately and represent replications in time.

Experimental control fish, fish receiving bacterial antigen, and fish receiving viral antigen were held in separate tanks. The fish were fed live or chopped forage fish and chopped beef liver once a day, but were not fed for about 24 hr before blood samples were taken.

The fish were weighed periodically and their general health was monitored by close observation and preparation of external wet mounts and smears for bacteria and parasites. Mortalities were examined for bacterial and parasitic pathogens. Sex of all fish was determined by examination of gonadal tissue at the termination of each trial.

A water flow of 3.79 l/min was maintained throughout the experiment; constantly aerated water from the Leetown National Fish Hatchery spring was used. The water supply had a total hardness of 262.5 ppm ($CaCO_3$), 100.0 ppm calcium (EDTA titration), and 3.04 ppm magnesium (Warren 1963). Water temperature in the holding facility was held between 27 and 28 C. Dissolved oxygen ranged from 6.5 to 7.8 ppm. When virus was used the effluent from the facility was treated with 200 ppm chloride for 2 hr and then neutralized with sodium thiosulfate before release.

Preparation of Antigens

Bacterial Antigen

Chondrococcus columnaris, strain BH-3, obtained from Dr. John Fryer, Oregon State University, was used throughout this research.

Test and immunizing antigens were prepared in Cytophaga broth (Anacker and Ordal 1959) on a rotary shaker at 200 rpm for 20 hr at 25 C, then centrifuged at 1400 × g for 45 min at 4 C, washed four times with physiological saline, and resuspended in physiological saline with 0.35% formalin. The antigen was standardized at 1.6×10^{10} cells/ml in a Petroff-Hausser counting chamber. The antigen was sonicated at 50 W for 40 sec with a Branson[2] microtip sonifier$_{(R)}$ converter and stored at 4 C.

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[2] Reference to trade name does not imply Government endorsement of commercial products.

Bacterial antigen for the microtiter agglutination test, grown as described, was centrifuged, washed, and resuspended in physiological saline with 0.35% formalin. The bacterial suspension was standardized at 2.1 × 10^9 cells/ml in a Petroff-Hausser counting chamber. The suspension was then sonicated at 70W for 30 sec to break up the bacterial clumps, refrigerated, and allowed to settle for 24 h[3] at 1.7 × 10^6 cells/ml of essentially monodispersed bacterial cells, labeled, and stored at 4 C for later use. This suspension was diluted 1:5 with physiological saline at the time it was used for microtiter agglutination tests.

The *C. columnaris* antigen for use in immunoelectrophoresis and immunodiffusion was placed in a dry ice ethanol bath and sonicated at 70 W for 2 min in 20 sec increments. The sonicate was then centrifuged at 500 × g for 20 min to remove large particles of bacterial cell debris and concentrated four-fold on an Amicon concentrating cell fitted with a PM-10 filter and operated at 9.9 kg/.000645 m^2.

American Type Culture Collection strain VR 665 CCV was used as the viral antigen. Stock virus was inoculated onto 90% confluent monolayer cultures of Certified Cell Line 59 (brown bullhead) in Blake bottles, cultured in Eagle's minimal essential medium with 10% fetal bovine serum at a pH of 7.4 to 7.8, and incubated at 25 C. The virus was harvested when cell sheet destruction was complete and centrifuged at 300 × g to remove cellular debris.

The supernatant fluid was then centrifuged at a median value of 54,500 × g for 30 min. The resulting virus pellets were resuspended in Hanks' balanced salt solution (HBSS), titrated by plaque assay, and diluted to contain about 10^6 plaque forming units (PFU) per milliliter. The quantified virus was then placed in sterile screw cap vials and stored at -80 C.

Viral antigens for immunodiffusion and immunoelectrophoresis were grown and harvested as previously described and subjected to three treatments:

1) The harvest from one Blake bottle culture was resuspended in 5 ml of HBSS at a concentration of 1 × 10^8 PFU/ml and sonicated for 1 min at 70 W in a dry ice ethanol bath to disrupt the virions. An Amicon concentrating cell with a PM-10 filter operated at 9.9 kg/.000645 m^2 was then used to achieve two- (1 × $10^{8.5}$), four- (1 × $10^{8.8}$), and six-fold (1 × 10^9) concentrations of the sonicate. These final concentrations were used as immunodiffusion antigens.
2) The harvest from a second Blake bottle culture was resuspended in 5 ml of HBSS, sonicated at 70 W for 10 sec to disrupt the viral conglomerates, concentrated five-fold, and used for immunodiffusion as viable virions.
3) The harvest from a third Blake bottle was concentrated to contain approximately 1 × 10^{10} virions/ml and extracted with Tween 80 and ether as recommended by Fink et al. (1969). Tween 80 was added to the concentrated virus in the amount of 0.1% by volume. The mixture of Tween 80 and virus was gently agitated for 5 min and a volume of ether equal to that of the virus mixture added. This mixture of virus, Tween 80, and ether was also gently agitated for 5 min; the ether allowed to separate from the viral mixture for 5 min and then aspirated (Robert Naegle, personal communication). The ether-extracted viral antigen was used on standard 1% Noble agar plates and slides, as well as on special slides and plates that had an additional 2 mm thick layer of 0.5% agar.

Inoculation Procedure

Blood samples were taken from all fish before inoculation, and the serums tested for either *C. columnaris* or CCV preimmunization antibody.

Each test fish receiving bacterial antigen was injected i.m. with a mixture of 0.1 ml of bacterial cells (1.6 × 10^{10} cells/ml) in 0.1 ml FCA, which was followed 7 days later with a similar injection. During the same time periods, each control fish received an i.m. injection of 0.1 ml diluent combined with 0.1 ml FCA.

Each experimental fish receiving viral antigen was injected i.p. with 296 PFU of CCV in 0.2 ml HBSS, followed by a similar injection 7 days later. Control fish received i.p. injections of 0.2 ml HBSS.

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[3] Technique recommended by Takahisa Kimura, Hokkaido University, Hokkaido, Japan, personal communication.

Once the average titer of each test group had reached a high level, I gave a single secondary antigen injection to study the secondary response.

Blood samples were taken from Group I experimental and control fish at 2, 4, 6, and 9 wk post-initial injection and 1½, 3, and 6 wk after the secondary injection.

Blood samples where taken from Group II and Group III experimental and control fish at 1, 2, 3, 5, 7, and 9 wk after the initial injection and 1½, 3, and 6 wk after the secondary injection.

Channel Catfish Serums

I took trial blood samples of about 4 ml from each fish at every bleeding by entering the hemal arch in the caudal peduncle area with a 22-guage needle. The blood, collected in Vacutainer (R) tubes, was allowed to clot at room temperature (T°) for 2 to 3 hr before it was centrifuged at 400 × g for 20 min at 4 C. The serum was then removed with a tuberculin syringe, placed in a sterile 2 ml glass vial, labeled, and stored at -20 C until tested. All serum samples were handled on an individual basis. A portion of each serum from the 2 and 3 wk blood samples from catfish Groups II and III was not immediately frozen, but was stored at 4 C and used for acrylamide gel protein separation and enzyme visualization.

After antibody titration of all serum samples and the acrylamide gel analysis of the 2 and 3 wk samples, high titered serums were selected for use in antibody characterization. All of these serums were kept at -20 C and were not pooled until the various antibody characterization tests were performed.

Rabbit Antiserums

Rabbit antiserums were prepared by inoculation of young, New Zealand white rabbits of about 1.2 kg live weight. All rabbit blood samples were held at T° for 4 h before the clot was removed. The supernatant liquid was then centrifuged at 400 × g for 10 to 15 min at 4 C to remove any remaining cells. The clear serum was then transferred to sterile containers and stored at -20 C.

All rabbits were injected according to the following schedule:

1) A total of 3 ml of antigen in FCA was injected i.m. into each flank and subcutaneously on the abdominal wall of each rabbit, four different times, at 10-day intervals.
2) A trial bleeding of 10 ml of blood was taken on the 50th day from each rabbit and precipitin bands were developed by immunodiffusion on Ouchterlony plates. Since excellent precipitin lines formed in all trials, the rabbits were exsanguinated by cardiac puncture on the 52nd day.

Human IgG (Microbiological Associates, Bethesda, Maryland) reconstituted in distilled water at a concentration of 10 mg IgG/ml was combined with an equal volume of FCA and injected into two rabbits.

Pooled normal channel catfish serum was combined with an equal volume of FCA and injected into three rabbits by using the same procedure described.

Estimate of Channel Catfish Antibodies

Bacterial agglutination titers of anti-*C. columnaris* serums were determined with the microtiter system detailed in the microtiter manual of Cooke Engineering Co., Alexandria, Va.

Channel catfish virus was quantified and serum levels of neutralizing activity were assayed by the two-phase plaque assay of Wolf and Quimby (1973).

Acrylamide Gel Electrophoresis

In electrophoresis in acrylamide gels, I followed the general methods developed by Davis (1964). The sampling gel was omitted, however, and the sample placed directly on the spacer or stacker gel and overlaid with a 5% sucrose solution. Standard Tris-Glycine buffer, pH 8.3, was used in both chambers, with a bromphenol blue tracker dye in the upper buffer. Gels were prepared in Canalco standard glass gel tubes, #163348, using 1.2 ml of 7% acrylamide separating gel and 0.2 ml large pore spacer gel. Final separating gel column length was 60 mm and stacking gel 8 mm.

A Canalco Disc electrophoresis chamber, model 1200, was used with a Beckman Duostat power supply. Samples were electrophoresed at 4 C using 1 milliampere per column until the tracking dye had entered the column (30 to 45 min) and then at 3 or 4 milliamperes per column until the tracking dye was about 2 mm from the bottom of the tube (1½ h). The pH of the spacer and separating gels for lactic acid dehydrogenase (LDH) enzymography was adjusted to pH 5.6 with HCl and the separating gel was also modified to contain 7.5% acrylamide.

Serum samples of 25 μl were used for protein separation and samples of 50 μl for isozyme separations. All cytochemical substrates were purchased from Sigma Chemical Company, St. Louis, Mo.

Electrophoretic runs were standardized by preparing one lot of buffer for all determinations on a single enzyme system, preparing all of the gel stock solutions at the same time for analysis of each enzyme, and carefully measuring the quantity of gel placed in the gel tubes.

Total protein content of each serum from the 2- and 3-wk blood samples was determined by the Folin technique (Lowry et al. 1951) to standardize the amount of protein (i.e. enzyme) placed on the gels for electrophoresis. Avoidance of possible enzyme changes from accumulative anemia was overcome by selecting serums from the 2- and 3-wk blood samples for enzymography.

Each electrophoretic run included normal control serum from a previous run to monitor reproducibility of the enzymograms. Gels were examined for possible differences due to sex, then isozymes of normal serums were compared with those of experimental fish. Comparisons were also made during each electrophoretic run between representative normal, antibacterial, and antiviral serums before the results were combined. Protein and isozyme bands were conventionally numbered, beginning with those of highest mobility, closest to the anode (Markert and Moller 1959; Hunter and Burstone 1960; Allen et al. 1965; Goldberg 1965b; and Hochachka 1966). A small letter following the numeral designated a band which occurred in less than 50% of the gels.

Reagent controls were provided for each enzyme system, which included columns that lacked enzyme substrate. The acrylamide gel comparisons for proteins and each enzyme system were based on 45 zymograms, 15 from each class of serum.

Visualization of Protein and Enzyme Activity

Total proteins were stained with Coomasie brilliant blue in trichloracetic acid (Maurer 1971). The gels were placed in the staining mixture for at least 6 h and were subsequently destained in 7% acetic acid.

Isozyme banding patterns for acid phosphatase (AcP), alkaline phosphate (AlP), esterase (Es), LDH, and Maleic dehydrogenase (MDH) were determined by the methods in Enzyme Analysis by Canalco (Anonymous 1964) with minor modifications.

The enzyme activity of acid phosphatase was determined by the pararosaniline hydrochloride-sodium alpha naphthol acid phosphate method with a modified gel incubation of 1 h at T°. The gels were stored in 1N sodium hydroxide for 2 to 6 days.

Alkaline phosphatase isozyme activity was visualized by the sodium alpha napthol acid phosphate method and modified for an incubation time of 1 h at T°.

Alpha napthyl acetate and fast blue BB (diazonium salts) were used for Es visualization. The incubation was timed for 1 h at T°, and the gels were stored in 7% acetic acid.

Exploratory enzymography showed the superiority of lithium dl-lactate over the recommended sodium dl-lactate for LDH staining. An incubation period of 2 h at T° proved satisfactory for enzyme visualization. The gels were rinsed four times with distilled water after incubation and stored in 7% acetic acid. They were stained and stored in the dark.

Cytochrome oxidase isozyme activity was visualized by the method of Maurer (1971) with an incubation time of 1 h at T°.

The results of the acrylamide gel electrophoresis of channel catfish isozymes and serum proteins were evaluated by comparing electrophoretic migration of the bands and by noting the presence or absence of bands and the staining intensity of the bands.

Electrophoretic migration was assessed by calculating the relative mobility (Rm) of each band, which was the distance of band migration from the origin (top of the running gel) divided by the total electrophoretic migration of the bromphenol blue dye front. The bands of each gel were measured in their storage tubes by using a metric rule and diffuse fluorescent back lighting as an aid in visualizing the sites of minor enzyme and protein activity.

Antibody Characterization

Heat Sensitivity

Samples of immune catfish serum were removed from storage and allowed to warm to room temperature. Anti-CCV serum was diluted to 1:100 and anti-*C. columnaris* serum to 1:4 with Hanks' BSS. Vials (4 ml capacity) were equilibrated to each test temperature in a constant temperature waterbath (Precision Scientific Co., serial #11-W-12), after which a 1-ml sample of antiserum was added, the vial was capped, and the material held at each test temperature for 30 min. Test temperatures ranged from 30 to 70 C in 10 C increments, and one sample was brought to 100 C. The serums were rapidly cooled in ice water after heat incubation. The test was replicated 1 wk later.

Reduction and Alkylation of Antibody

Samples of normal and immune catfish serums were pooled for reduction and alkylation by a procedure similar to that of Cisar (1972). All pooled serums were diluted 1:10 in tris-HCl buffered saline (TBS) at pH 7.2 before treatment. Reduction was performed by dialysis for 24 h at T° against 100 volumes of 0.1 M 2-ME, 0.1 M 2-ME in 2 M urea or 0.1 M 2-ME urea or 0.1 M 2-ME in 6 M urea. Each of these solutions was prepared in TBS at pH 7.2. The reduced samples were then placed in 100 volumes of 0.02 M iodoacetamide in tris buffered saline at pH 7.2 and aklylated by dialysis for 48 h at T°. The iodoacetamide mixture was changed after the first 24 h of alkylation. After alkylation, all samples were dialyzed against four changes of TBS, 500 volumes each, at T° for 48 h. Unreduced samples were dialyzed against TBS, 2 M urea in TBS, and 6 M urea in TBS as procedural controls.

Sephadex G-200 Column Chromatography

Sephadex G-200 column chromatography was used to determine the approximate molecular weight of channel catfish immunoglobulin. Gel filtration medium preparation was that of Pharmacia Fine Chemicals, Piscataway, N. J. The column was loaded with 1.5 ml of undiluted catfish antiserum with an anti-bacterial titer of 1:2048 or an anti-CCV serum titer of about 76% plaque neutralization at a serum dilution of 1:100. Separation of both channel catfish anti-*C. columnaris* and anti-CCV immunoglobulins by Sephadex G-200 was replicated. The flow rate was about 15 ml/h, and 2 ml fractions were collected with Isco Golden Retriever automatic fraction collector. The fractions were assayed for protein concentration by measuring optical density at 280 nm in a Beckman DU-2 spectrophotometer. Each fraction was titrated by using plaque neutralization for anti-CCV or microtiter agglutination for anti-*C. columnaris*.

DEAE Column Chromatography

Ion-exchange chromatography was carried out according to the procedure of Trump (1970) on anion exchange resin, DEAE-Sephadex A-25. For each run, 5 ml of whole, pooled, and undiluted antiserum was used. The anti-*C. columnaris* serum had a titer of 1:1024 and the anti-CCV serum had a plaque neutralization titer of 76% at a serum dilution of 1:100. Chromatography was done in a Siliclad (R) coated column (2.6 × 66.0 cm) at 4 C with a flow rate of 15 ml/h and a 30 cm hydrostatic head. Fractions of 5 ml were collected with an Isco automatic fraction collector and assayed for protein concentration by measuring optical density at 280 nm in a Beckman DU-2 spectrophotometer. Fractions were then examined for bacterial titer or viral plaque neutralization, by methods previously described.

Molecular Weight Analysis

Molecular weight studies were conducted by using continuous sucrose gradients (10 to 40% wt/vol) prepared in the apparatus designed by Britten and Roberts (1960). Stock sucrose solutions were prepared with 0.075 M TBS at pH 7.2. Beckman cellulose nitrate tubes (2.54 × 7.62

cm) were used to make gradients of 25 ml each, which were stored at 4 C overnight before use.

Partially purified DEAE antibody fractions of anti-*C. columnaris* and anti-CCV serums were used for the molecular weight determinations. Protein markers were 5% solutions of trypsin (mol w 24,000), bovine serum albumin (mol w 67,000), and a 2% solution of human gamma globulin (mol w 150,000) in 0.75 M TBS at pH 7.2. Protein markers and catfish antibody samples were centrifuged for 12 h at 4 C in a Beckman model L ultracentrifuge at a median value of 41,400 × g with a SW 25.1 rotor. A fractionator similar to that designed by Martin and Ames (1961) was used for the collection of 1 ml fractions from the bottom of the tubes. Each fraction was tested for protein by absorption at 280 nm in a Beckman DU-2 spectrophotometer. Fractions which contained protein were dialyzed against three changes of TBS at pH 7.2 for 48 h at 4 C to remove the sucrose, and then concentrated to 2 ml in an Amicon pressure dialysis cell for later assay of antibody activity.

Immunodiffusion and Immunoelectrophoresis

Immunodiffusion was carried out in 60 mm plastic culture dishes by double gel diffusion in 1% Noble agar as described by Ouchterlony (1968). In each test well, 0.1 ml of antigen or antiserum was used.

Immunoelectrophoretic analysis (IEA) was performed by the slide technique (Nowotny 1969), with the Gelman immunoelectrophoresis apparatus.

Channel catfish serums were electrophoresed for 1 to 1½ h at T° with prechilled buffer and a constant current of 200 V. Undiluted antiserums and concentrated antigens were employed for development of precipitin arcs and were incubated in a moist chamber for 24 to 48 h. The slides were then photographed directly or washed, dried, and stained with amido black as described by Uriel (1971).

RESULTS

Experimental Fish

The fish had an average weight of 265 g (range, 135–463 g) at the beginning of the experiment. The fish remained healthy, and there were no indications of abscesses at the injection sites. Anemia that developed in some fish was attributed to repeated blood sampling. Two fish died from this anemic condition 9 wk postinoculation.

Kinetics of Antibody Response

Immune Response to *Chondrococcus columnaris*

The primary response of channel catfish to the bacterial antigen was rapid and strong (Fig. 1). The secondary response was also rapid but weaker than that which often occurs in warmblooded animals. All fish injected with *C. columnaris* antigen mounted a fairly strong immune response with a minimal agglutination titer of 1:1024 (Figs. 2, 3, 4).

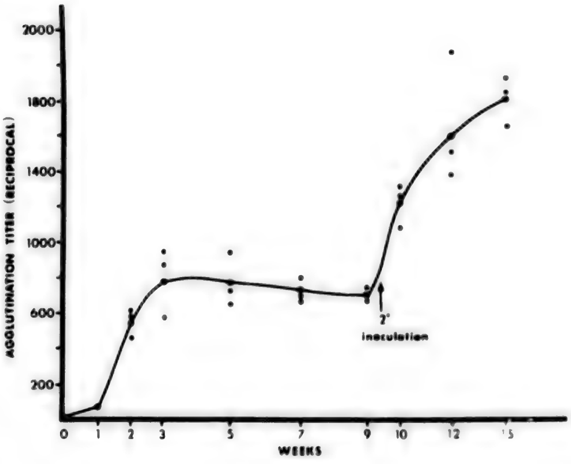

Fig. 1. Immune response of channel catfish to primary and secondary inoculation with *C. columnaris* antigen. Curve shows mean value of all data; points show means for each of three trials.

Antibody production was apparent 1 wk after vaccination, with an average titer of 1:65 (range 1:8–1:256) (Fig. 1). The highest average titer of 1:796 (range 1:256–1:2048) occurred 3 wk after primary injection and then began to gradually decline after 5 wk until secondary injections were given at 9½ wk. Secondary injection of bacterial antigen at 9½ wk resulted in a rapid titer increase to an average of 1:1214 (range 1:512–1:2048) 1½ wk after secondary injection.

The average anamnestic response continued to increase for 6 wk after secondary injection (Fig. 3). The titer of the majority of the experimental fish began to stabilize 3 wk after injection (Figs. 2, 3, 4). The maximum average anamnestic response reached a level of more than twice that of the primary response, and some fish showed a secondary response six times greater than the primary response. The serums of preinoculation and control fish contained variable but low titers that did not exceed 1:16 throughout the experiment.

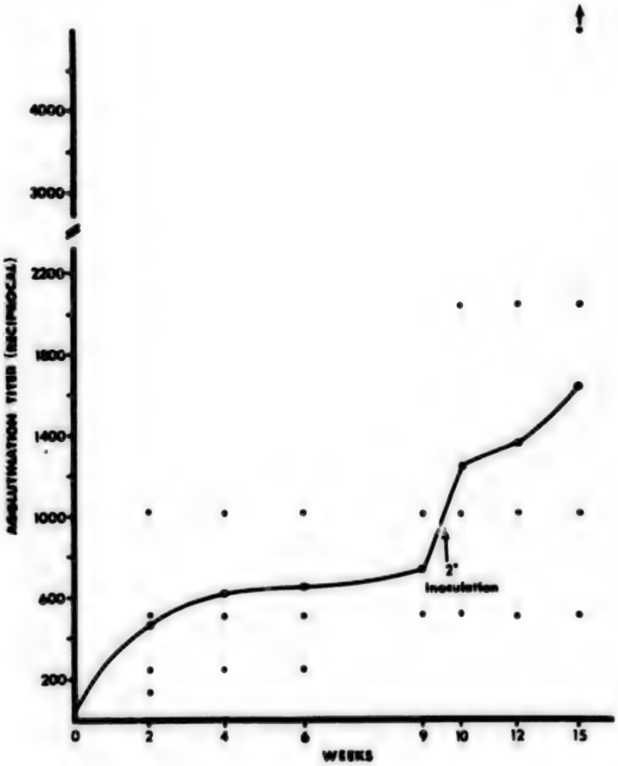

Fig. 2. Immune response of Group I channel catfish to primary and secondary inoculation with *C. columnaris* antigen. Curve shows mean value for the group; points show values for individual fish.

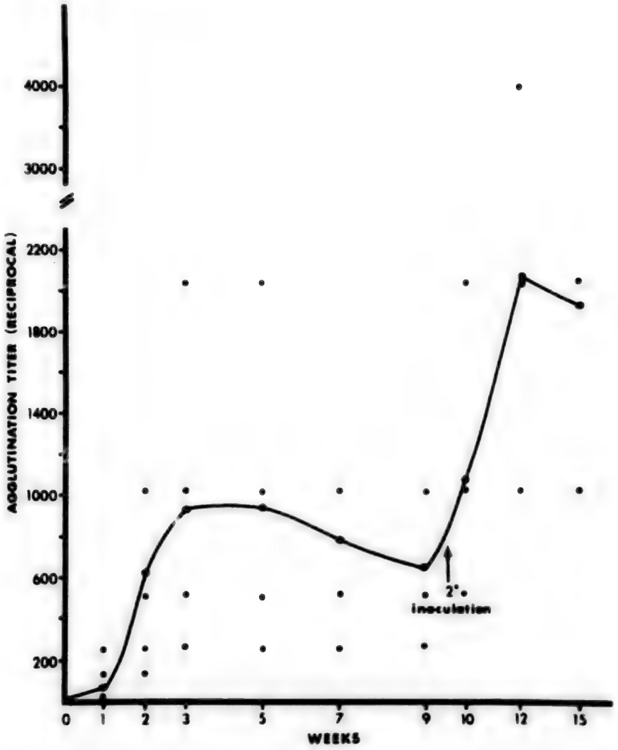

Fig. 3. Immune response of Group II channel catfish to primary and secondary inoculation with *C. columnaris* antigen. Curve shows the mean value of the group; points show response of individual fish.

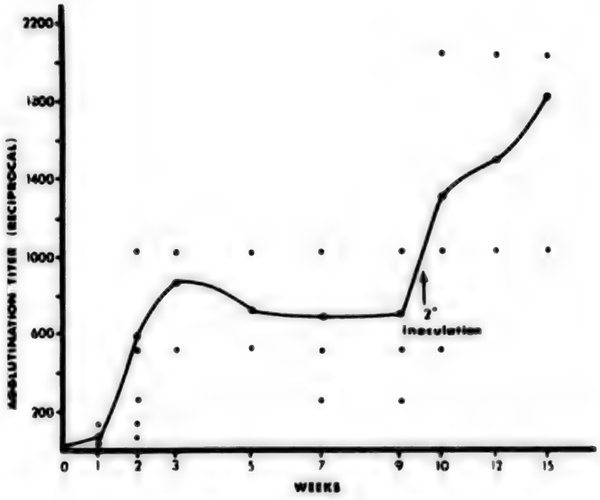

Fig. 4. Immune response of Group III channel catfish to primary and secondary inoculation with *C. columnaris* antigen. Curve shows the mean value of the group; points show response of individual fish.

Immune Response to Channel Catfish Virus

The primary immune response of channel catfish to CCV was highly variable (Fig. 5). Antibody titers were detectable in less than 50% of the individuals 1 wk after inoculation. The secondary response was also variable, weak, and relatively short (Figs. 6, 7, 8).

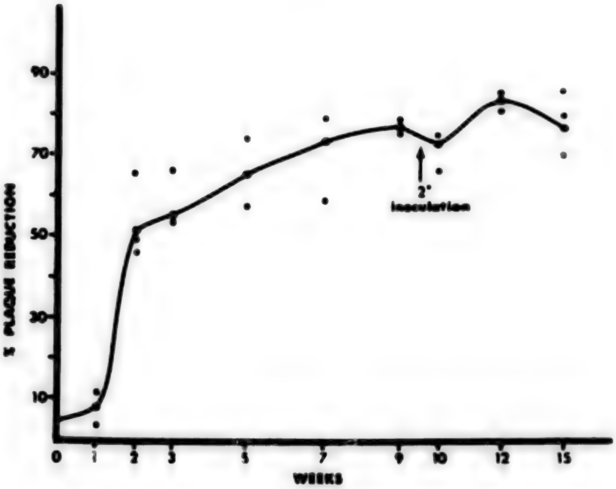

Fig. 5. Immune response of channel catfish to primary and secondary inoculation with CCV. Curve shows mean values for all data; points show means for each of three trials.

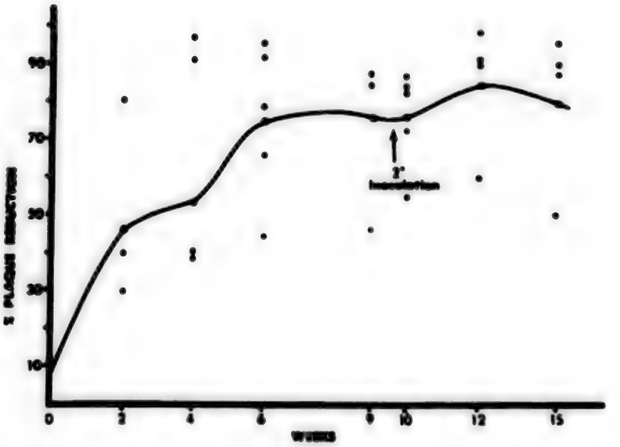

Fig. 6. Immune response of group I channel catfish to primary and secondary inoculation with CCV. Curve shows mean values for the group; points show response of individual fish.

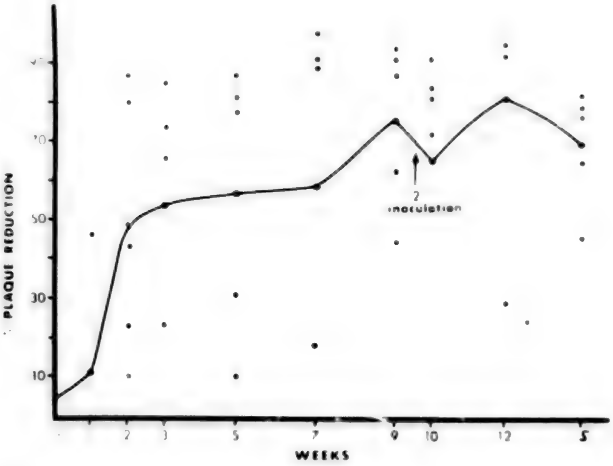

Fig. 7. Immune response of Group II channel catfish to primary and secondary inoculation with CCV. Curve shows the mean value of the group; points show response of individual fish.

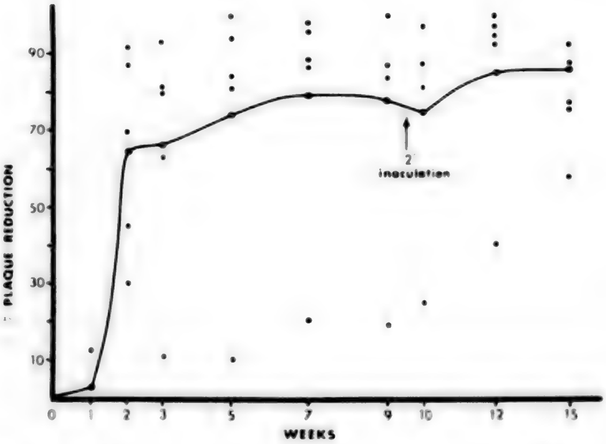

Fig. 8. Immune response of Group III channel catfish to primary and secondary inoculation with CCV. Curve shows the mean value of the group; points show response of individual fish.

Antibody was detectable in 1 wk in only 3 of 10 test fish in Groups II and III (Figs. 7, 8). Mean titers were highest after 9 wk when the plaque neutralization value was 77% (range, 19–100%) at an antiserum dilution of 1:100, with about 100 PFU of virus. A gradual decline of neutralization values began the 9th wk after injection. An anamnestic response was not detectable until 1½ wk after the secondary injection, and the plaque neutralization titers had begun to decrease 6 wk after the secondary injection (Figs. 6, 7, 8). The secondary response was slight (increase over the peak average primary titer was only 7%) and of short duration (titer decreased 40 days after the secondary injection).

The ability of individual fish to produce antibody against the viral antigen varied tremendously among individual fish. Six test fish exhibited a primary plaque neutralization value greater than 75% at 2 wk whereas 3 other fish did not exceed 50% plaque neutralization during the entire 9 wk study of the primary response (Figs. 6, 7, 8). Five of the 15 viral test fish demonstrated small preimmunization titers (range, 10-25%). All except one of the control fish remained without neutralizing activity throughout the experiment.

Acrylamide Gel Electrophoresis

Protein Visualization

Experimental fish inoculated with bacterial or viral antigen had a strikingly noticeable site of major activity at position 4a which was absent in control fish (Fig. 9). This band was of minor activity in bacterial serum samples and stained lavender, whereas in viral serum samples it was of major activity and stained dark blue.

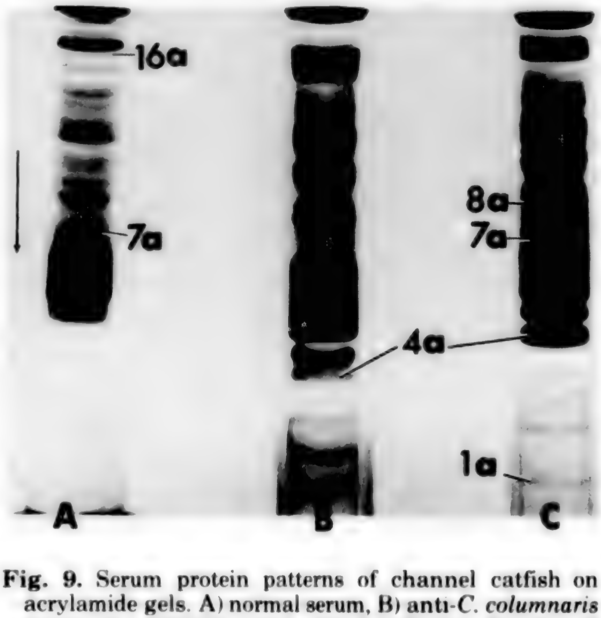

Fig. 9. Serum protein patterns of channel catfish on acrylamide gels. A) normal serum, B) anti-*C. columnaris* serum, C) anti-CCV serum. Arrow indicates direction of electrophoretic migration. Note additional bands at positions 1a, 4a, and 8a in experimental fish, absence of band 7a in anti-bacterial serum samples and absence of band 16a in experimental fish.

Band 16a, present on normal serum gels, was absent in all fish receiving bacterial or viral antigen. A minor band at position 7a in 50% of normal and viral serum samples was not present in bacterial test fish. The rapidly moving fractions at site 1a, and band 8a, were present in 30% of the viral serum samples but were lacking in normal and bacterial serum samples. A majority of the protein fractions from fish inoculated with viral antigen exhibited greater relative mobility than did comparable protein fractions from normal fish or those inoculated with bacterial antigen.

Resolution of normal catfish serum proteins on acrylamide gels differentiated 19 primary bands and 2 additional secondary bands (7a and 16a) which occurred in less than 50% of the serum samples. The protein fractions differed in staining coloration from blue through pale lavender. Bands 5, 6, 7, and 17 exhibited major activity. The most rapidly moving components, bands 1 through 4, were typified by their pale lavender color.

To field test the results obtained with experimental fish, protein patterns of 25 Leetown National Fish Hatchery channel catfish brood fish with variable CCV serum neutralizing activity were examined on acrylamide gels. There was no consistent correlation between the appearance of band 4a and CCV serum neutralization levels in the brood stock.

Acid Phosphatase Isozymes

Serums from 50% of the fish receiving bacterial antigen and 30% of the fish injected with virus contained a slowly migrating band (4a) of major activity which was not present in normal catfish serums (Fig. 10). Band 3 showed less enzymatic activity in experimental fish than in the controls but had an increased Rm. Normal channel catfish serum acid phosphatase was resolved into 4 amber isozyme bands. Fractions 1, 2, and 3, the most rapidly moving, were of intermediate mobility and at a site of minor activity, whereas fraction 4, a slowly moving band, occupied a site of major activity.

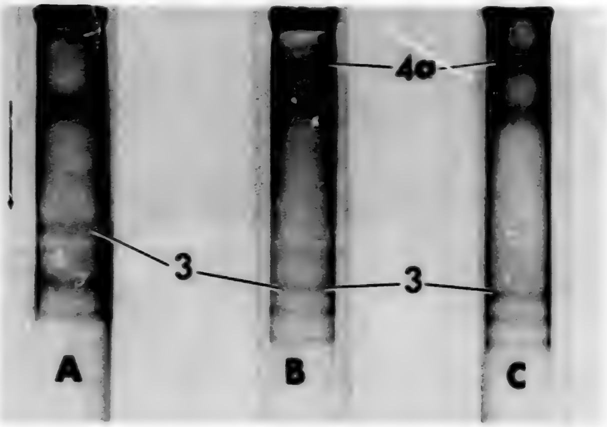

Fig. 10. Zymograms of channel catfish serum acid phosphatase. A) normal serum, B) anti-*C. columnaris* serum, C) anti-CCV serum. Arrow indicates direction of electrophoretic migration. Note increased migration of band 3 and addition of band 4a in the experimental fish.

Alkaline Phosphatase Isozymes

Differences in the alkaline phosphatase isozyme patterns between normal catfish serums and serums from fish injected with bacterium or virus were relatively minor (Fig. 11). All fish receiving bacterial antigens had 4 bands, whereas 10% of the normal fish lacked band 3 or 4. None of the 4 bands were always present in virus injected fish; bands 1, 2, and 4 were lacking in 30% of the fish and band 3 in 10%. The Rm values for serum AlP bands were slightly, but consistently, greater in control fish than in fish receiving bacteria or virus. The AlP bands stained amber, were of slow mobility, and occupied sites of minor activity in normal and experimental fish.

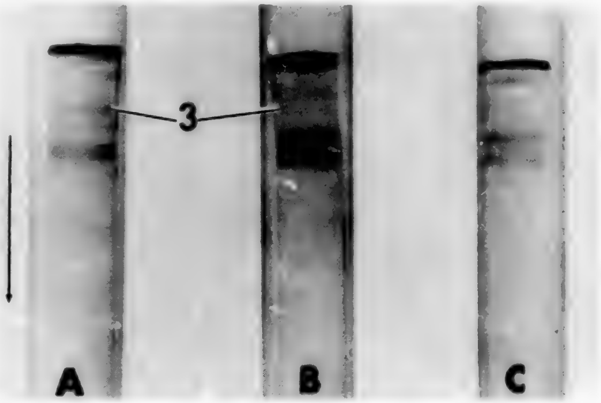

Fig. 11. Zymograms of channel catfish serum alkaline phosphatase. A) normal serum, B) anti-*C. columnaris* serum, C) anti-CCV. Arrow indicates direction of electrophoretic migration. Note absence of band 3 in anti-CCV serum.

Cytochrome Oxidase Isozymes

Relatively small differences existed between the Rm values or percentage of occurrence of the isozyme bands between normal and test fish (Fig. 12). Band 1b occurred in 20% of the serum samples from fish injected with virus but was absent in samples from control fish and fish inoculated with bacteria. Band 2a occurred in 10% of the serum samples from normal fish and fish injected with bacteria and in 40% of the samples from fish receiving virus. Band 5 appeared in 70% of the normal serum samples, 80% of the samples from fish injected with bacteria, and 50% of the samples from fish receiving virus. Normal catfish serum cytochrome oxidase was resolved into 5 primary bands and 2 secondary bands at positions 1a and 2a which occurred in less than 50% of the gels. All 7 bands were of minor enzyme activity and stained purplish-blue.

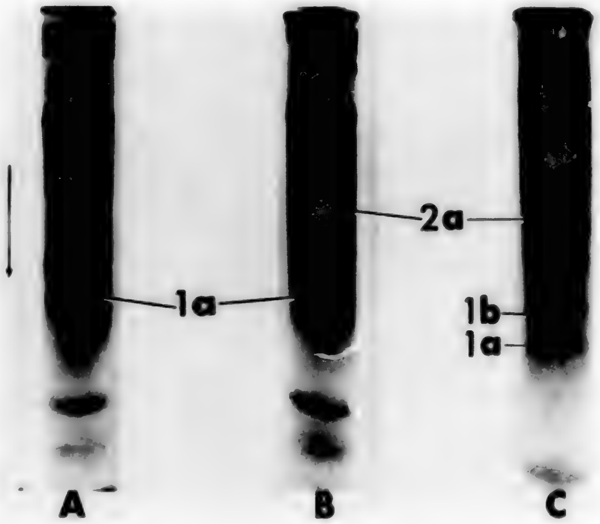

Fig. 12. Zymograms of channel catfish serum cytochrome oxidase. A) normal serum, B) anti-*C. columnaris* serum, C) anti-CCV serum. Arrow indicates direction of electrophoretic migration. Note band 1b in anti-CCV serums, band 2a in both anti-bacterial and anti-viral serums, and the slight increase in relative mobility of band 1a in viral fish.

Esterase Isozymes

Serum samples from 10% of the fish injected with bacteria and 30% of those injected with virus contained 2 rapidly moving esterase fractions (1a and 1b) which were not present in serums from normal fish (Fig. 13). All of the rapidly moving esterase fractions occurred at sites of major enzyme activity, and bands 2, 3, and 4 had consistently greater Rm values in fish receiving bacteria or virus than in normal fish. Serums from normal and test fish also contained 6 primary black esterase bands and a secondary band at site 3a.

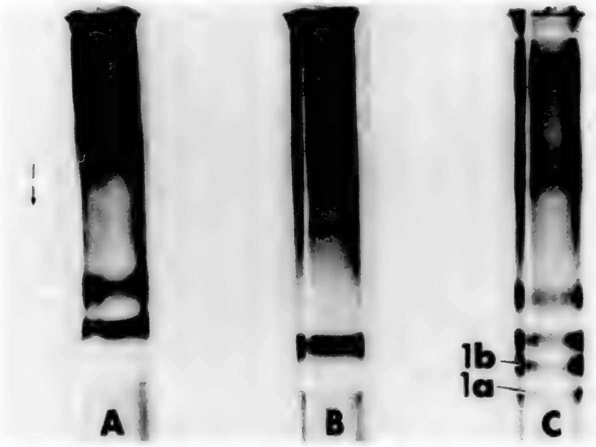

Fig. 13. Zymograms of channel catfish serum esterase. A) normal serum, B) anti-*C. columnaris* serum, C) anti-CCV serum. Arrow indicates direction of electrophoretic migration. Anti-CCV zymogram contains additional bands (1a and 1b).

Lactic Acid Dehydrogenase Isozymes

Serums from fish receiving bacterial antigens or virus had two poorly separated bands of intensified maximum enzyme activity at sites 4 and 5, and 3 more rapidly moving bands of minor activity which were only faintly visible (Fig. 14). In contrast the 4 deep purple LDH isozyme bands were of slow to intermediate mobility in normal catfish, but were clearly separated sites of major enzyme activity.

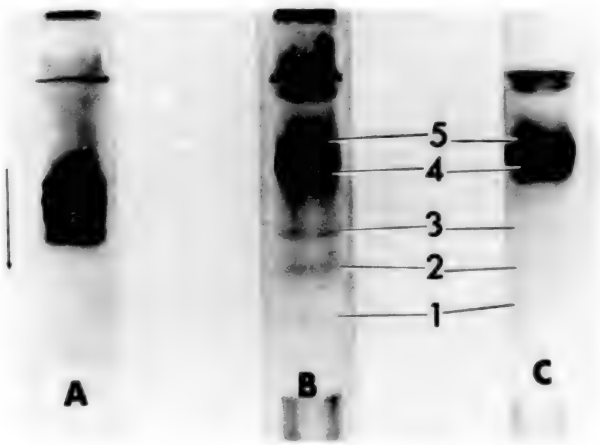

Fig. 14. Zymograms of channel catfish serum lactic acid dehydrogenase. A) normal serum, B) anti-*C. columnaris* serum, C) anti-CCV serum. Arrow indicates direction of electrophoretic migration. Note difference in staining intensity and relative mobility of experimental fish serums versus normal serum (bands 1-5).

Malic Acid Dehydrogenase Isozymes

The response to the test antigens resulted in only minor differences in MDH isozymes between serums from normal fish and those from fish injected with bacteria or virus. MDH isozymes were resolved into 12 purple bands; bands 1-3 occupied sites of minor enzyme activity, and bands 4-7 sites of major activity (Fig. 15). The Rm values for bands 3-8 were consistently greater for serums from the experimental fish than for serums from the controls.

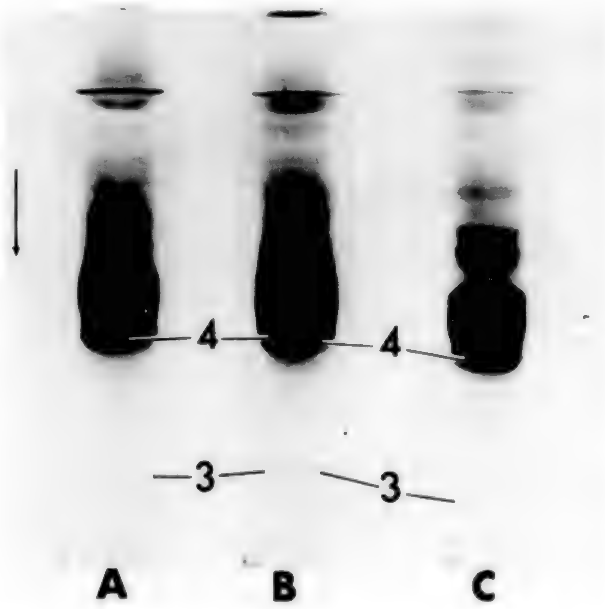

Fig. 15. Zymograms of channel catfish serum malic acid dehydrogenase. A) normal serum, B) anti-*C. columnaris* serum, C) anti-CCV serum. Arrow indicates direction of electrophoretic migration. Note minor enzyme activity at site 3 and major enzyme activity of band at site 4.

Antibody Characterization

Heat Stability

Exposure of channel catfish antiserums to heat resulted in a loss of antibody activity at temperatures below those recommended for treatment of mammalian serums.

Preliminary trials with 5 anti-CCV serum samples at 1:100 dilution showed an average loss of 25% of the specific antibody activity when serums were inactivated at 56 C for 30 min. Replicate heat inactivation curves demonstrated the effect of various temperatures on channel catfish antibody against *C. columnaris* and CCV (Figs. 16, 17). Anti-*C. columnaris* antibody activity was unaffected at temperatures up to 40 C, but 50% of the original antibody activity was lost at 50 C, 66% at 60 C, and more than 95% at 70 C (Fig. 16).

Catfish anti-CCV whole serum showed loss of specific antibody activity of 16% at 50 C, 36% at 60 C, and more than 95% at 70 C (Fig. 17).

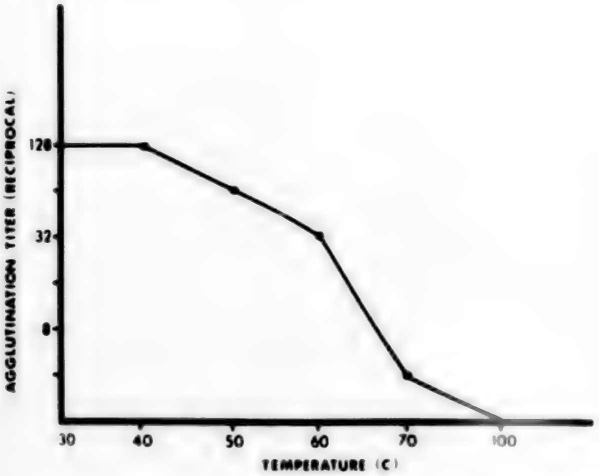

Fig. 16. The effect of temperature (30-min exposure) on agglutination activity of channel catfish anti-*C. columnaris* serum.

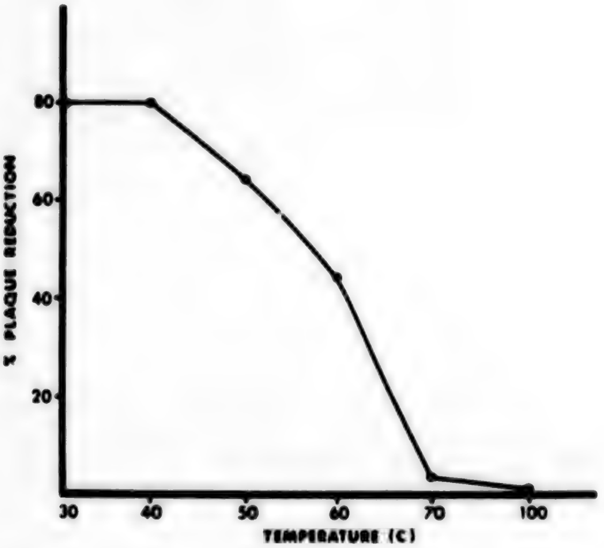

Fig. 17. The effect of temperature (30-min exposure) on plaque neutralization activity of anti-CCV serum.

Reduction and Alkylation of Antiserums

Partial reduction of catfish antiserums with 0.1 M 2-ME resulted in a 50–75% loss of the anti-*C. columnaris* activity (Fig. 18) and a 35–55% loss of the anti-CCV activity (Fig. 19). The ratio of 2-ME resistant activity to total activity did not increase with time for either antiserum. Exposure of the catfish antiserums to 0.1 M 2-ME and 2M urea resulted in a 92–98% reduction of the anti-*C. columnaris* activity and a 90–100% inactivation of the anti-CCV activity. The loss of antibody activity was complete when catfish antiserums were exposed to 0.1 M 2-ME and 6 M urea.

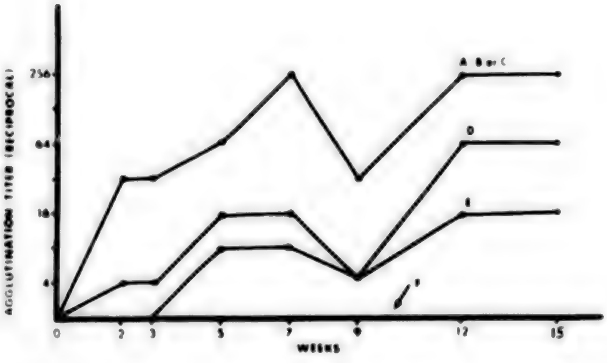

Fig. 18. Effects of partial reduction and alkylation on the activity of anti-*C. columnaris* serums. A) untreated antiserum, B) antiserum exposed to 2 M urea, C) antiserum exposed to 6 M urea, D) antiserum exposed to 2-ME, E) reduced and alkylated antiserum with 2-ME and 2 M urea, F) reduced and alkylated antiserum with 2-ME and 6 M urea.

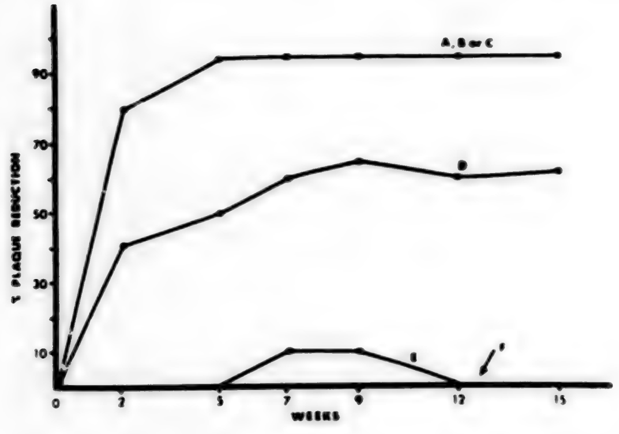

Fig. 19. Effects of partial reduction and alkylation on the activity of anti-CCV serums. A) untreated antiserum, B) antiserum exposed to 2 M urea, C) antiserum exposed to 6 M urea, D) antiserum exposed to 2-ME, E) reduced and alkylated antiserum with 2-ME and 2 M urea, F) reduced and alkylated antiserum with 2-ME and 6 M urea (activity completely lost).

Sephadex Column Chromatography

Separation of pooled whole catfish antiserums by column chromatography on Sephadex G-200 resulted in 3 distinct protein peaks (Figs. 20, 21). Titration of the fractions indicated that antibody activity angainst both *C. columnaris* and CCV was contained entirely in the excluded volume and was thus associated with the macroglobulin fraction of the catfish serum (mol wt > 200,000).

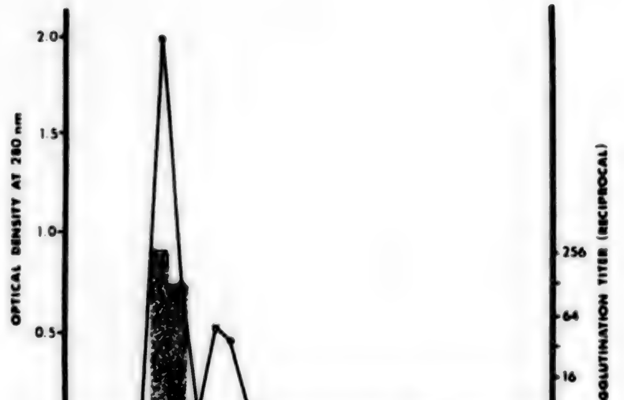

Fig. 20. Elution diagram of channel catfish anti-*C. columnaris* serum from Sephadex G-200. Shaded area shows fractions containing antibody activity.

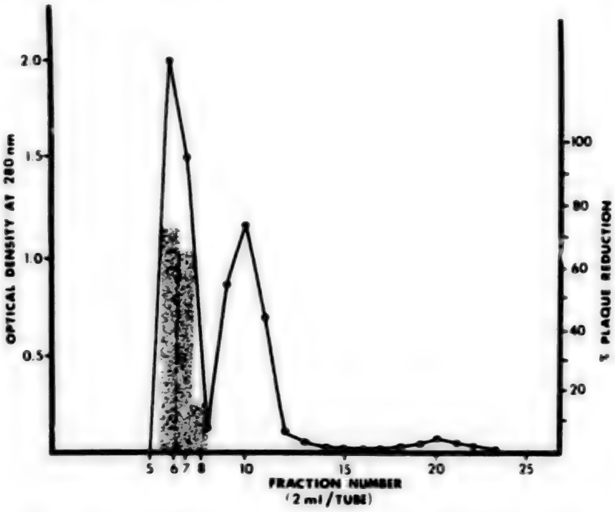

Fig. 21. Elution diagram of channel catfish anti-CCV serum from Sephadex G-200. Shaded area shows fractions with antibody activity.

DEAE Column Chromatography

The fractionation of whole catfish antiserums on the anion exchange resin DEAE Sephadex A-25 separated the antibody into two populations. The antibody activity was located within the second and third serum protein peaks after release of the Tris-HCl limited buffer in 0.3 M NaCl. Maximum anti-*C. columnaris* activity occurred in fractions 22-23 and 38-40 with a microtiter agglutination of 1:64 (Fig. 22). Anti-CCV activity was greatest in fractions 24-26,

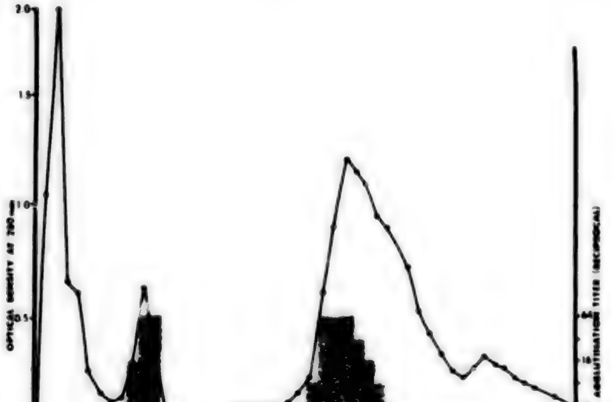

Fig. 22. Chromatogram of channel catfish anti-*C. columnaris* serum from a DEAE Sephadex A-25 anion exchange column. Shaded areas indicate fractions containing antibody activity.

which had a plaque neutralization titer of 100%, and was also evident in fractions 54–63, which had a plaque neutralization titer of 44–62% (Fig. 23).

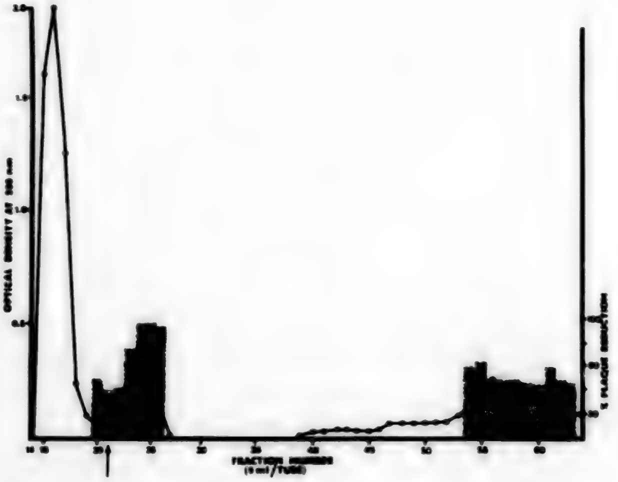

Fig. 23. Chromatogram of channel catfish anti-CCV serum from a DEAE Sephadex A-25 anion exchange column. Shaded areas indicate fractions containing antibody activity.

Molecular Weight Analysis

Antibody activity was found in all fractions which contained catfish protein (Fig. 24). The relationship of the molecular weight of known protein markers to catfish immunoglobulins gave a molecular weight estimate of 800,000–900,000 daltons for specific catfish anti-*C. columnaris* and anti-CCV immunoglobulins (Fig. 25).

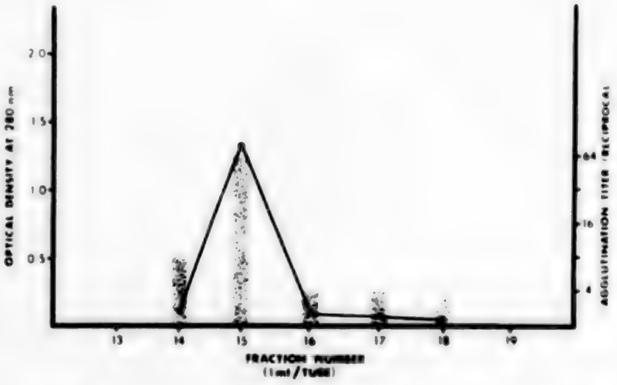

Fig. 24. Sucrose density gradient ultracentrifugation of pooled channel catfish antiserum from DEAE Seohadex A-25 anion exchance column. Note that antibody activity (shaded areas) coincided with protein activity.

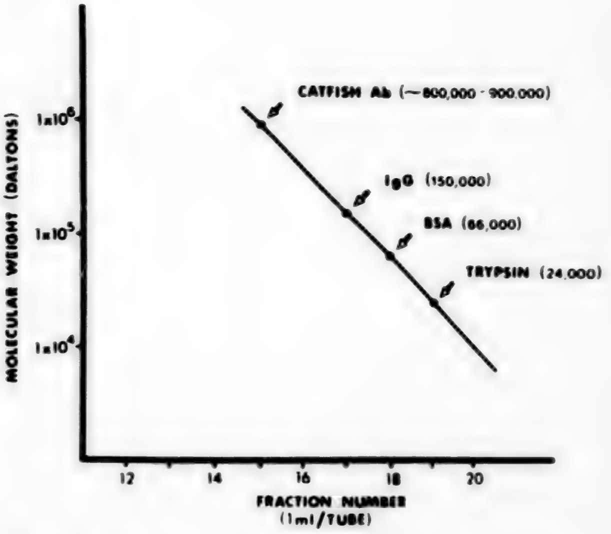

Fig. 25. Sucrose density gradient ultracentrifugation of protein molecular weight markers and catfish antibody from DEAE Sephadex A-25 anion exchange column.

Immunodiffusion

An Ouchterlony double gel diffusion comparison of various catfish serums with anti-*C. columnaris* activity revealed that serum precipitins were first detectable 5 wk after primary antigen injection (Fig. 26). Early antiserums (5 wk) formed two precipitin lines of complete identity with late primary response antiserums and with antiserums from the

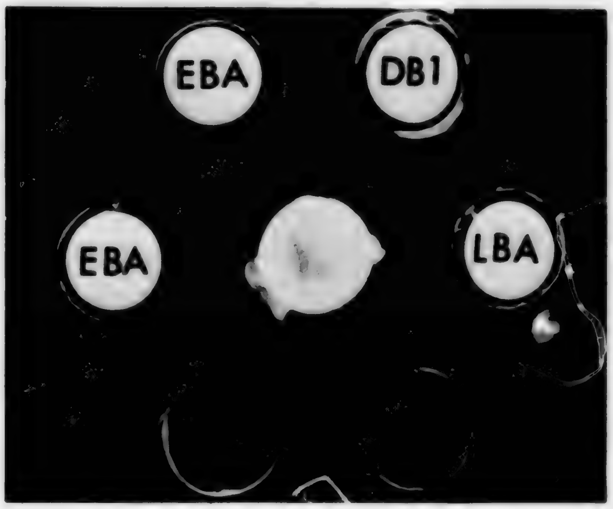

Fig. 26. Ouchterlony analysis of channel catfish anti-*C. columnaris* serums with *C. columnaris* antigen; EBA, early bacterial antiserum (3 and 5 wk); DB1, DEAE bacterial antibody population 1; LBA, late bacterial antiserum (9 wk).

secondary immune response at 12 wk (Figs. 26, 27). In addition, late primary and all secondary antiserums contained an additional antigenic component which was not present in early anti-*C. columnaris* serums (Figs. 26, 27).

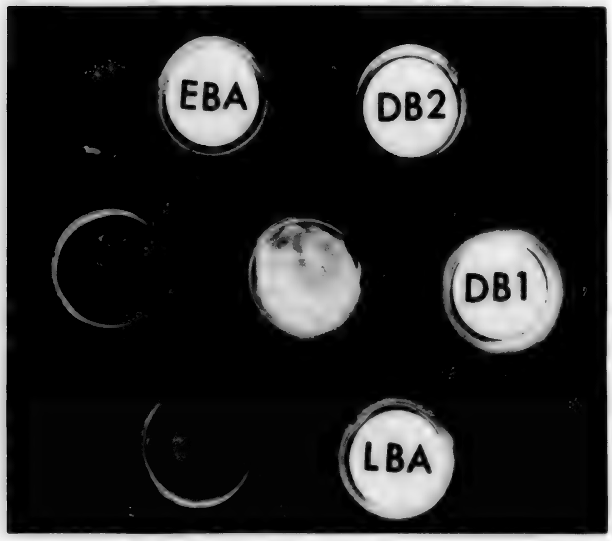

Fig. 27. Ouchterlony analysis of channel catfish anti-*C. columnaris* serums with *C. columnaris* antibody; EBA, early bacterial antiserum (5 wk); DB2, DEAE bacterial antibody population 2; DB1, DEAE bacterial antibody population 1; LBA, late bacterial antiserum (12 wk).

Catfish antibody was separated on DEAE ion-exchange column chromatography into two separate populations—DB-1 and DB-2. These populations formed two lines of complete identity with each other, but population DB-1 had an additional component (Fig. 27). Population DB-1 also formed three lines of identity with components of the late, 9 wk (Fig. 26) and secondary, 12 wk (Fig. 27) antiserums. Population DB-2 formed one precipitin band that had complete identity with early (3 wk) antiserums and one that had partial identity with early antiserums (Fig. 27). A third component was contained in late antiserums (9 wk) and secondary antiserums that was not present at 3 wk.

These results indicated that all catfish anti-*C. columnaris* serums have at least two antigenically distinct components which react with the specific antigen and which can be separated by their ionic charge differences.

Double diffused anti-CCV serum formed precipitin bands only on 0.5% agar plates with ether-Tween extracted viral antigen concentrated to about 1×10^{10} PFU/ml (Fig. 28). Sonicated CCV antigen and intact virions produced no precipitin bands on 0.5 or 1.0% agar plates. Viral precipitin lines were much weaker and more diffuse than those of the bacterial system. Very faint viral precipitins could be detected in anti-CCV serums 2 and 3 wk after the primary injection (Fig. 28). The precipitin band was slightly more intense after 5 wk and became readily visible in all subsequent serum samples (Fig. 28).

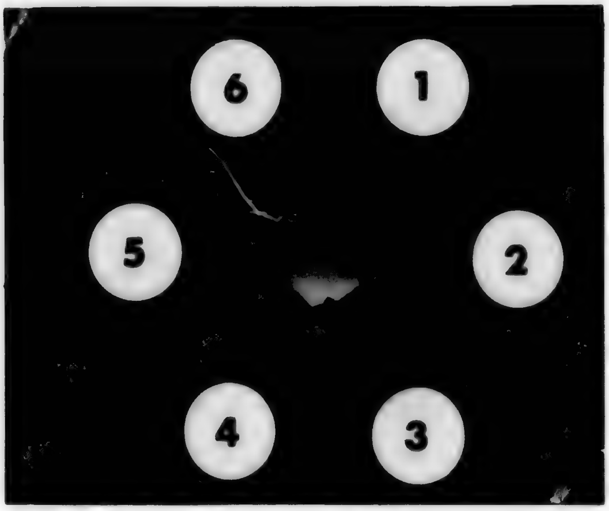

Fig. 28. Ouchterlony analysis of channel catfish anti-CCV serums with CCV antigen; 1) early viral antiserum (2 wk); 2) early viral antiserum (3 wk); 3) early viral antiserum (5 wk); 4) late viral antiserum (7 wk); 5) late viral antiserum (9 wk); 6) late viral antiserum (12 wk).

Late anti-CCV serums displayed two antigenic components as compared to the three components of late anti-*C. columnaris* serums. Two components were not readily visible when whole anti-CCV serums were compared with DEAE anti-CCV serum populations. No detectable difference in CCV precipitins was observed between antibody populations one and two separated on DEAE column chromatography (Fig. 29); both populations formed lines of complete identity with early and late CCV antiserums.

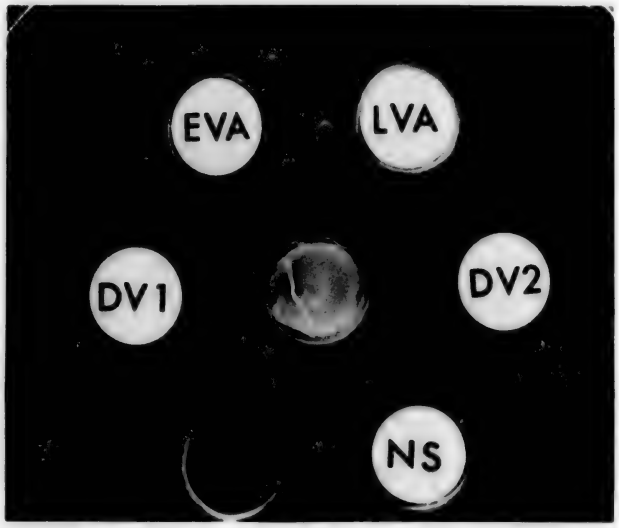

Fig. 29. Immunodiffusion of channel catfish anti-CCV serums with CCV antigen; NS, normal catfish serum; DV1, DEAE viral antibody population 1; EVA, early viral antibody (5 wk); LVA, late viral antibody (9 wk); DV2, DEAE viral antibody population 2.

Immunoelectrophoresis

Normal whole catfish serum was resolved into nine protein components after immunoelectrophoresis and development with rabbit anti-normal catfish serum (Fig. 30). The four major serum components which migrated in the anodal direction resemble the albumins and alpha-globulins of human serum in their electrophoretic mobility. The two components which showed maximum mobility toward the cathode resemble the beta- and gamma-globulins of human serum as described by Kabat (1968).

Fig. 30. Immunoelectrophoresis of channel catfish normal serum with rabbit anti-normal channel catfish serum. Arrow denotes component of gamma electrophoretic mobility.

When normal serum was compared with anti-*C. columnaris* immune serum (Fig. 31), the difference in precipitin arcs formed by the albumins and beta-globulins observed in carp by Sorvachev et al. (1962) and Gulyaev et al. (1966) was not detected. However, the serum component of gamma-globulin-like mobility did show an increased intensity related to the duration of immunization.

Fig. 31. Immunoelectrophoresis of channel catfish anti-*C. columnaris* serum (5 wk) with rabbit anti-normal channel catfish serum. Note increased cathodal mobility of the gamma component (arrow).

Immunoelectrophoresis of channel catfish anti-*C. columnaris* serums developed with sonicated *C. columnaris* antigens showed that early antiserums contained immunoglobulins in one gamma-globulin-like precipitin arc (Fig. 32).

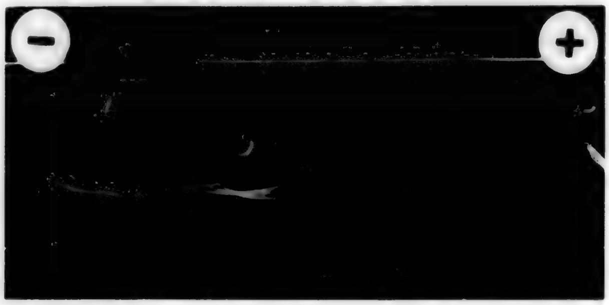

Fig. 32. Immunoelectrophoresis of channel catfish anti-*C. columnaris* serum (5 wk) with sonicated *C. columnaris* antigen (upper trough) and rabbit anti-normal channel catfish serum (lower trough). Arrow indicates position of the specific anti-*C. columnaris* immunoglobulin arc.

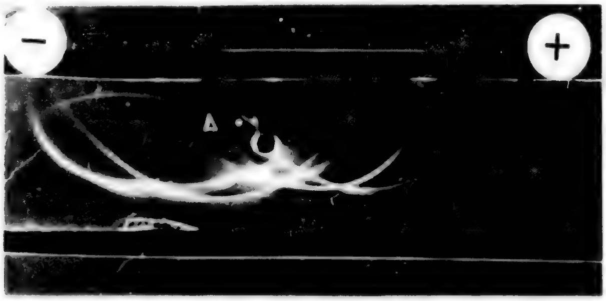

Fig. 33. Immunoelectrophoresis of channel catfish anti-*C. columnaris* serum ([illegible] wk) with sonicated *C. columnaris* antigen (upper trough) and rabbit anti-normal channel catfish serum (lower trough). Arrow indicates position of second immunoglobulin arc.

A second immunoglobulin arc was first visible 9 wk after injection (Fig. 33). In subsequent samples, this second immunoglobulin arc increased in length and strength and had mobility similar to that of a beta-globulin. The initial immunoglobulin arc continued to become longer and more pronounced in both the primary and secondary responses and definitely appeared to have the mobility of a gamma-globulin (Fig. 34).

Anti-CCV immunoglobulin arcs were obtained only with concentrated ether-Tween extracted viral antigen on agar slides with a 0.5% agar overlay. A viral precipitin arc was faintly visible at 5 wk and increased in intensity with time of immunization (Fig. 35). Only one precipitin arc was detected with anti-CCV immunoglobulins which showed slight mobility in the anodal direction and, unlike the anti-*C. columnaris* antibodies, appeared to have the mobility of an alpha or beta globulin.

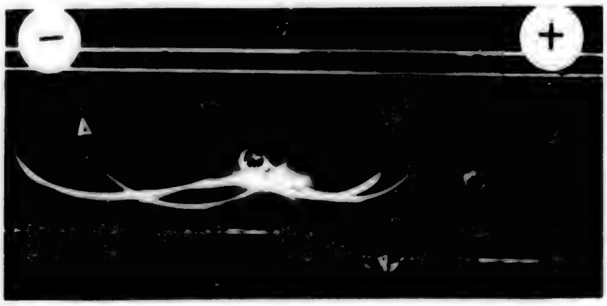

Fig. 34. Immunoelectrophoresis of channel catfish anti-*C. columnaris* serum ([illegible] wk) with sonicated *C. columnaris* antigen (upper trough) and rabbit anti-normal channel catfish serum (lower trough). Note increased cathodal migration of immunoglobulin arc (arrow).

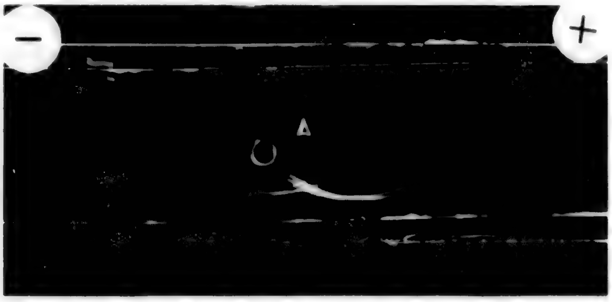

Fig. 35. Immunoelectrophoresis of channel catfish anti-CCV serum ([illegible] wk) with CCV antigen (center trough) and rabbit anti-normal channel catfish serum (lower trough). Arrow indicates position of specific anti-CCV immunoglobulin arc.

DISCUSSION

The present study on the immunization of channel catfish was separated into three general subjects: 1) studies on the immune response, 2) evaluation of serum protein and isozyme systems with disc electrophoresis, and 3) the partial characterization of channel catfish antibodies.

Immune Response

The immune response is generally thought to be slower in fish than in mammals, because most fish immunology has been carried out on coldwater species at low temperatures. In the present study of a warmwater species, the channel catfish responded to both *C. columnaris* and CCV antigens 1 wk after primary injection. This rapid response corroborates a series of works on warmwater fishes: Frenzel and Ambrosius (1971), Goncharov (1962), Papermaster (1964), Shelton and Smith (1970), Trump and Hildemann (1970), Watson et al. (1968), and Vladimirov (1972).

The multiplicity of envrionmental, genetic, physiological, and experimental design factors involved in the immune response in any one test limits the value of comparisons between fish immunization experiments, especially with regard to peak titers and the critically important aspect of protective immunity. However, the time interval involved in obtaining an initial response or a mean peak titer is of some comparative value if the experimental conditions are well defined.

Channel catfish injected with *C. columnaris* antigen in the present study responded strongly with a minimal agglutination titer of 1:1024 and had peak titers similar to those found at 25 C by other workers in carp and goldfish (Frenzel and Ambrosius 1971, Shelton and Smith 1970, Trump and Hildemann 1970, and Watson et al. 1968).

The secondary response of channel catfish immunized with *C. columnaris* was rapid but weaker than that in warmblooded animals. Avtalion (1969) and Trump and Hildemann (1970) reported similar weak secondary responses in carp and goldfish.

Low *C. columnaris* agglutination activity was observed throughout the experiment in control fish. The persistence of these low titers in serums of all catfish before immunization, and in the control channel catfish, suggested that these were nonspecific natural agglutinins. Cushing and Durall (1957) found isoagglutinins in brown bullheads and channel catfish and Wiener et al. (1968), Kuhns et al. (1969, 1972), and DiConza (1970) all reported nonspecific natural agglutinins with low levels of activity in catfishes.

Serums from 20 Leetown National Fish Hatchery channel catfish brood stock combined with *C. columnaris* antigen also had low bacterial agglutination titers.

Although the initial immune response of the experimental catfish to CCV was initiated at about the same time as the response to bacteria, the subsequent course of antibody formation differed considerably. This difference was predictable because the bacterial responses depended on injection of an initial amount of bacteria in FCA sufficient to produce a measurable response without the addition of bacteria at a later time, whereas, the response to CCV depended on the replication of the virus.

The immune response to CCV was dependent on infection of the fish by the small amounts of viable virus injected and replication of the virus. Therefore, the initial peak titer after viral injection would be expected to require a longer time than that after the bacterial injection, and the secondary response to CCV would be expected to be weaker and shorter. The time to peak initial titers in the present study was similar to the 60 days observed by Plumb (1973b), who also injected channel catfish with viable CCV. Catfish in Plumb's study also elicited a rapid secondary response, not much greater than the initial response and of short duration.

In contrast to the response of all individual catfish receiving *C. columnaris* antigen, 3 of 15 fish injected with viable CCV mounted very low responses, probably due to either a reduced immunological competence or insufficient infection by the pathogen. A variable response to viable CCV in catfish was observed by Plumb (1973b), who also noted that the injection of catfish with heat-killed virus produced no detectable immune response.

The response to *C. columnaris* and CCV in the present study was similar to that observed by McGlamery et al. (1971) who immunized catfish with BSA and vesicular stomatitis virus (VSV). The response to BSA was rapid and strong but the response to VSV, although detectable in 2 wk, was of low titer and the antibody level declined rapidly. These results are predictable; a high titer could not be expected because VSV did not replicate in channel catfish and the immune response was strictly dependent on the minuscule quantity of antigen given. In addition, the antibody was partly inactivated by treatment at 56 C for 30 min.

Poor secondary response to viruses may also result from lack of antigen recognition. Clem and Sigel (1966) hypothesized that secondary stimulation with viral antigens probably causes antigen-antibody complexes, but that these complexes may not be recognized as foreign.

The rather insignificant secondary response of channel catfish to CCV may have been due to the presence of specific viral antibody. Rowley and Fitch (1964) found that rats often failed to show noticeable response to low viral antigen doses when homologous circulating antibody was already present.

Serum Protein and Isozyme Systems

Certain obvious differences in total protein, AcP, Es, and LDH banding patterns between normal and immunized catfish on acrylamide gels suggested that further research might yield useful advances toward developing disease diagnostic methods. Many of the gel comparisons of isozymes did not yield clearcut answers, however, and minor changes in the isozyme systems—particularly differences in Rm values and changes in bands of minor activity—will not be of value until more data are accumulated.

The advanced isozyme technology of human clinical medicine points out the tremendous possibilities of the technique. Limited funds, the high cost of equipment, and the great variability in results have deterred research on the technique as applied to fish diseases. Fish pathobiologists should be able to make limited use of serum analysis on acrylamide gels, however, even if only obvious or gross pattern charges are detectable.

Protein and isozyme analysis will require establishment of "normal" patterns for economically important cultured fish species before this technique can be widely used. Thurston (1967), who investigated the influence of genetics and stress on rainbow trout serum protein patterns, stated a "normal" pattern could not be established, and recommended extreme caution in interpreting the results of serum protein changes.

Bell (1968) stated that clinical enzymology is usually of little value without knowledge of "normal" or "standard" values. He reported sufficient changes in the SGOT levels to distinguish trout infected with kidney disease from healthy trout, but the degree of specificity of the SGOT change has not been determined in fish. Amend (1973) observed a plasma LDH pattern change in rainbow trout infected with IHNV and believed it was specific for the disease. However, a similar change of LDH was noted in the present study after channel catfish were inoculated with CCV and *C. columnaris*.

In addition to the establishment of normal gel patterns for fishes, field testing of fishes with natural infections will be necessary before widespread use of enzymography can be made. The CCV suspect brood stock examined in the present experiment did not offer conclusive results. The development of the same protein changes in some of the brood fish as those shown by experimental CCV fish offered encouragement. The absence of a direct correlation between anti-CCV serum titers and serum protein changes in the brood stock may not negate the usefulness of the technique. Correlation between anti-CCV serum titers and carrier state of the virus has not been determined. Many isozyme systems in addition to those used in the present study should be monitored.

Establishment of "normal" gel patterns will be only one of the problems involved in protein and isozyme analysis. Replication of results is also dependent on extreme care in gel preparation; quantification of amount of protein analyzed; selection of gel porosity and pH; and selection of suitable temperature, amperage, and substrate.

The gel system which resulted in excellent results with brook trout LDH (Goldberg 1965a) was totally unsuitable for resolution of channel catfish LDH. Some success was achieved after 3 mo by altering gel pore size and pH as suggested by Gould (personal communication). Results were often visible to the unaided eye, but were not of sufficient intensity to be clearly shown in photographs.

Background staining and rapidly fading isozyme bands often interfered with the photographic representation of LDH, MDH, and cytochrome oxidase. Because the cytochrome oxidase and LDH isozyme systems were extremely unstable, freezing of serums resulted in a loss of isozyme activity, as did electrophoresis at T° and storage of serums for a week at 4 C.

Characteristics of Channel Catfish Antibodies

Results of the exposure of catfish immune serum to varying temperatures from 30–100 C for 30 min periods clearly illustrated the thermolabile nature of the antibody. Exposure of anti-CCV to 50 C for 30 min resulted in a loss of 16% of the viral neutralizing activity and 50% of the anti-*C. columnaris* antibody activity.

Cisar (1972) found that coho salmon anti-*Aeromonas salmonicida* antibody was heat labile. Fifty percent of the agglutination activity was lost from exposure to 50 C for 30 min and 75% was lost at 55 C for 30 min. These results strongly indicate that specific antibody activity in the serum of many fishes is lost at temperatures routinely used for inactivation of nonspecific activity in warmblooded animals.

The reduction of antibody activity after exposure to 2-ME resulted from the cleavage of covalent bonds. Further reduction of antibody activity by 2 M urea and total loss by exposure to 6 M urea indicated that residual antibody resulted from either noncovalent bonding or from exposure of additional covalent bonds to reduction by 2-ME. My results differ from those of Ambrosius et al. (1967) who reported the complete destruction of brown bullhead antibody activity by partial reduction and alkylation.

There was no indication that the ratio of 2-ME resistant activity to total activity increased with time. The failure of a shift toward the production of 2-ME resistant antibodies during the present study, corroborated similar findings by several other investigators (Ambrosius et al. 1967; Kuhns and Chuba 1968; Cisar 1972; and Evelyn 1971). Uhr et al. (1962), however, did observe an antibody shift.

The separation of pooled whole catfish antiserums into three protein peaks by Sephadex G-200 column chromatography was similar to separations effected by Marchalonis (1971) with antiserums from goldfish and carp. The fact that the catfish antibodies were macroglobulins, contained in the excluded fraction, also agrees with the results of several other investigators. Hodgins et al. (1967) and Dorson (1972) recovered rainbow trout antibodies in the excluded fraction. Coho salmon immunoglobulins to *A. salmonicida* were macroglobulins (Cisar 1972), as were goldfish and carp antibodies (Marchalonis 1971, and Everhart 1972). No attempt was made to further purify the channel catfish macroglobulins separated by G-200 Sephadex chromatography. DEAE column chromatography was chosen as a means of separating the antibodies from other serum proteins by net charge. Whole anti-*C. columnaris* and anti-CCV serums were both separated into two antibody populations by DEAE column chromatography. Trump (1970) observed an almost identical separation of goldfish immunoglobulins into two populations by DEAE column chromatography.

An estimated molecular weight of 800,000 to 900,000 daltons for specific channel catfish immunoglobulins is similar to the results obtained with most teleosts (Uhr et al. 1962; Clem and Leslie 1969; Clem and Small 1970; Trump 1970; Marchalonis 1971; and Clem 1971). However, Acton et al. (1971) calculated the molecular weight of channel catfish macroglobulin to be only about 610,000.

Immunodiffusion and immunoelectrophoresis of whole and partially purified serums also demonstrated a heterogeneous immunoglobulin system that increased in complexity with length of immunization. These results support the separation of catfish immunoglobulins into two populations by DEAE column chromatography. Cisar (1972) also found heterogeneity with coho salmon anti-*A. salmonicida* immunoglobulins. Two antibody populations were also demonstrated in goldfish by Trump (1970), Trump and Hildemann (1970), and Everhart (1972), in which the earliest formed immunoglobulin precipitin arcs had beta mobility, and the arcs observed from fish immunized longer had IgG mobility.

The production of immunoglobulins of IgG mobility was also supported by the work of Uhr et al. (1962), Clem and Sigel (1963), Summerfelt (1966), and Evelyn (1971). The apparent discrepancies in the literature pertaining to the mobility of fish immunoglobulins can be partly explained in light of the findings in the present study. Channel catfish produced at least two immunoglobulins to *C. columnaris* that differed in electrophoretic mobility and in time of initial appearance, and both increased in cathodal electrophoretic mobility as the immune response progressed.

The electrophoretic mobility of anti-CCV immunoglobulins differed from those of anti-*C. columnaris*. Thus, channel catfish produced immunoglobulins of different electrophoretic mobility due to differences in both antigen and length of time of antigenic stimulation. Pike (1967) also found that the nature of the antigen can influence the kinds of antibodies produced.

Earlier reports that fish immunoglobulins have different electrophoretic mobilities were often one-time assessments, and were based on the use of many different antigens. Considering kinetics and antigen differences, the earlier reports need not be considered as contradictory.

In contrast to the bacterial precipitin system, catfish precipitins to CCV were weak and at no time had a mobility resembling that of a gamma-globulin. Two catfish anti-CCV immunoglobulin populations were separated by DEAE ion exchange chromatography, but these, unlike the different anti-*C. columnaris* populations could not be demonstrated by immunodiffusion or immunoelectrophoresis.

The single viral precipitin could be demonstrated only by using the method of Fink et al. (1969), which exposed the viral nucleoid antigens and employed a less dense medium for antigen and antibody migration. The catfish anti-viral precipitins are the first fish viral precipitins listed in the literature detected by the use of fish antibody and homologous antigen.

The only even remotely comparable viral precipitin reaction was that of Jørgensen (1973) who reacted trout anti-IPN serum with rabbit serum produced against trout anti-IPN serum. The difference between the systems is that Jørgensen demonstrated the precipitins by using a third component. The electrophoretic mobility of the catfish anti-CCV immunoglobulin was comparable with that of the trout anti-IPN reported by Jørgensen. He also noted that the intensity of the IPN immunoglobulin arc increased with postimmunization time, a condition noted with both the viral and bacterial precipitins in channel catfish.

Failures of researchers to demonstrate fish precipitins to viral antigens may be due to methods used and to the absolute quantity of antigen involved. Viral precipitins could not be demonstrated in the present study with whole virions or with the standard 1% agar on either immunodiffusion plates or immunoelectrophoresis slides. Researchers who failed to demonstrate fish viral precipitins did not use ether-Tween extracted virus nor reduced agar percentages.

The difference between the bacterial and viral precipitins in catfish is supported by the findings of Pike (1967) who observed that the nature of the antigen can influence the type of antibody produced. Channel catfish produced different immunoglobulins, based on immunoelectrophoresis, apparently due to the difference in antigens used.

SUMMARY AND CONCLUSIONS

1. The primary immune response of experimental channel catfish to injections of *Chondrococcus columnaris* antigen in Freund's complete adjuvant reached maximum titers after about 4 wk. The primary immune response to viable CCV antigen in Hanks' BBS reached maximum titers at an average of 9 wk.
2. The secondary response to *C. columnaris* antigen was both rapid and pronounced. Two of three experimental groups had not reached maximum secondary titers 38 days post secondary injection when the experiments were terminated. The secondary respose to CCV antigen was rapid but weak and of short duration.
3. Acrylamide gel electrophoresis resolved channel catfish serum into 21 protein components. Differences were detected between the serums of normal as compared to experimental fish in the number of serum components and in the relative mobility of the fractions.
4. Six isozyme systems were also examined by disc electrophoresis: acid phosphatase, alkaline phosphatase, cytochrome oxidase, esterase, lactic acid dehydrogenase, and malic acid dehydrogenase. Comparisons made of serum from normal versus inoculated fish yielded major differences in the bands of acid phosphatase, esterase, and lactic acid dehydrogenase. Many less obvious differences in staining intensity of minor components and relative mobility values were present. Acrylamide gel enzymography appears to offer promise in disease diagnostics.
5. All antibody activity in anti-*C. columnaris* and anti-CCV serums was associated with the macroglobulin fraction having a molecular weight greater than 200,000. The large molecule fraction of both anti-*C. columnaris* and anti-CCV serums was further separated into two antibody populations by anion exchange chromatography.
6. Partial reduction and alkylation of channel catfish anti-*C. columnaris* and anti-CCV serums significantly reduced antibody ac-

tivity; activity was further reduced by additional exposure to 2 M urea and terminated by exposure to 6 M urea. A shift toward the production of a 2-ME resistant population of antibodies was not observed in the primary or secondary response to *C. columnaris* or CCV antigens.

7. Heating immune serums for 30 min at 50 C resulted in a 50% loss of anti-*C. columnaris* and a 20% loss of anti-CCV antibody activity.
8. Channel catfish immunoglobulin had an estimated molecular weight of 800,000 to 900,000 daltons.
9. Precipitins were obtained with channel catfish immune serums to both *C. columnaris* and CCV antigens. The reaction between immune fish serum and viral antigen constitutes the first known report of fish viral precipitins.
10. Immunodiffusion and immunoelectrophoresis of antibacterial serums revealed a heterogeneous assemblage of immunoglobulins that increased in complexity with time after immunization. In channel catfish anti-*C. columnaris* serums, immunodiffusion revealed two antigenic components in early serums and three in late serums; immunoelectrophoresis revealed one component in early and two in late serums.
11. Specific humoral antibodies to *C. columnaris* antigen were contained in the electrophoretically least mobile protein components. Early anti-*C. columnaris* immunoglobulin exhibited beta to gamma mobility which gradually changed to a pattern typical for human gamma globulin by late immunization. The second immunoglobulin to appear had beta-globulin mobility.
12. In contrast to the mobility of the anti-*C. columnaris* antibodies, specific viral antibodies in catfish had an alpha-2-globulin mobility.
13. The results of partial antibody characterization indicate that specific channel catfish immunoglobulins are macroglobulins, with characteristics similar to those of human IgM and most fish immunoglobulins.

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